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GENERALIZED CYTOMEGALIC INCLUSION-BODY DISEASE ASSOCIATED WITH PNEUMOCYSTIS PNEUMONIA IN ADULTS

A REPORT OF THREE CASES, WITH WEGENER'S GRANULOMATOSIS, THROMBOTIC PURPURA, AND HODGKIN'S DISEASE AS PREDISPOSING CONDITIONS

BY

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(RECEIVED FOR PUBLICATION JULY 4, 1959)

Three cases of generalized cytomegalic inclusion-body disease (salivary virus disease) in adults are reported, bringing the number of published cases up to 34. The infection is very rare in adults although well known in infants. As is often found in infants with this disease, pneumonia due to *Pneumocystis carinii* was also present in each case.

The first patient had Wegener's granulomatosis, which presented with acute otitis media: a review of histological material obtained at mastoidectomy eight weeks before death showed that inclusion-body cytomegaly was already present then. Various antibiotics and prednisolone were given, and the lesions in the respiratory organs and the arteritis healed to a considerable extent. Renal failure, however, was progressive and led to death.

The second patient had thrombotic purpura and died after a few weeks' illness, during which oxytetracycline and hydrocortisone were given. Congenital absence of the spleen was found at laparotomy, which was performed with the object of doing a splenectomy. Focal cryptococcal pneumonia was present *post mortem*: six years before death a solitary cryptococcal granuloma of one lung had been treated by lobectomy.

The third patient had had Hodgkin's disease for 18 years. During the first 12 years the disease had the characteristics of the so-called indolent form (" Hodgkin's paragranuloma ") and it then passed into the typical form. Deep *x*-ray therapy and cytotoxic drugs were used during the course of the disease at various times, and streptomycin and tuberculostatic drugs were given because of intercurrent tuberculous meningitis which developed three months before death.

In all three cases it seems likely that the underlying disease, or the drugs used in its treatment, predisposed to cytomegalic inclusion-body disease and concomitant pneumocystis pneumonia by lowering the patients' resistance. Just as some unusual types of fungal and bacterial infections have become less rare since the introduction of certain drugs, including antibiotics and steroids, it is possible that cytomegalic inclusion-body disease and pneumocystis infection may also be met with oftener in adults, perhaps particularly as a complication of the use of these drugs in the treatment of diseases which are specially liable to interfere with the body's defences.

The adult type of cytomegalic inclusion-body disease occurs usually, if not in fact always, as a complication of some other disease or its treatment, and in a considerable proportion of the cases the lesions have been widespread and serious. These features distinguish the adult type from the infantile type of the disease, which is often no more than an incidental post-mortem finding without clinical significance.

The current editions of most textbooks of medicine and virology say little or nothing about this infection: it is possible to give only a short account of selected aspects here, but further information, particularly about the disease in

infancy, is to be found in the admirable monographs of Seifert and Oehme (1957) and of Nelson and Wyatt (1959), and in articles by Wyatt, Saxton, Lee, and Pinkerton (1950) and France (1951) among others.

The Viral Nature of the Infection

VonGlahn and Pappenheimer (1925) studied a case of generalized cytomegalic inclusion-body disease in an adult, and on cytological grounds put forward the theory that the disease was caused by a virus. A year later Cole and Kuttner (1926) successfully propagated the disease through several generations of guinea-pigs by intracerebral inoculation of cell-free filtrates originally obtained from salivary tissue of naturally infected animals. Work on other animals has shown that each virus is specific to the species from which it is isolated. The presence of a virus in tissue from human cases has been demonstrated only recently: Smith (1956) isolated a virus which causes the development of similar intranuclear inclusion bodies in tissue cultures of human fibroblasts, and is neutralized by the serum of infants with the disease and by that of their mothers. Parallel observations confirming these results were made by Rowe, Hartley, Waterman, Turner, and Huebner (1956). Studies by Minder (1953) suggested that the virus itself can be shown by electron microscopy as aggregates of spherules in the optically clear zone between the intranuclear inclusion body and the nuclear membrane.

The Inclusion Bodies

Two sorts of inclusion are found in the affected cells, intranuclear and intracytoplasmic (Fig. 1). Every infected cell contains an intranuclear inclusion body: in contrast, the intracytoplasmic inclusions seem to have been found only in epithelial cells, particularly those of the ducts of secretory glands. At different stages of maturation and ageing the intranuclear inclusion bodies differ considerably in appearance and in the relative proportions of their chemical constituents. The variation in chemical composition explains some of the apparently conflicting results of different observers when recording the staining reactions and histochemistry of the inclusions. At its most typical, when fully mature, the intranuclear inclusion body is separated by a clear zone from the nuclear membrane and the nucleus has a characteristic "owl's eye" appearance (Fig. 2). Although the whole affected cell is anything from twice to four or more times its normal size the cytoplasm increases in amount approximately in

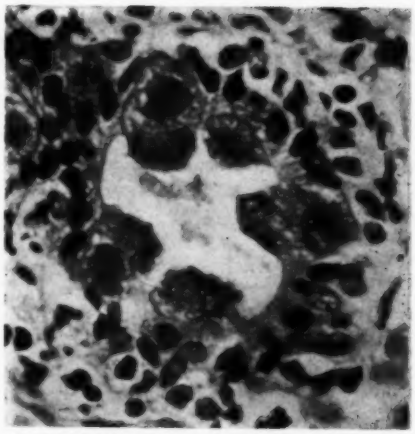

FIG. 1.—Inclusion-body cytomegaly affecting five cells of a parotid ductule (Symmers, 1960). The nucleus is seen in only three of the cells, and is much enlarged, consisting almost wholly of inclusion body without any of the clear zone of nucleoplasm which is characteristic at another stage in development of the infection (see Figs. 2 and 25). The nucleus is basally placed: the opposite pole of the cell protrudes into the lumen of the ductule, and intracytoplasmic inclusions are responsible for the darker appearance of this part. Haematoxylin-eosin. ×700.

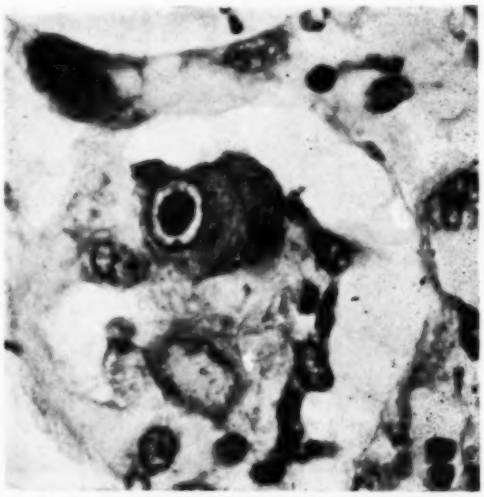

FIG. 2.—Case 1: Inclusion-body cytomegaly in a bronchial gland. "Owl's eye" appearance of the affected nucleus, with large central inclusion body separated by a clear zone from the nuclear membrane. A "polar body" on the nuclear membrane projects into the clear nucleoplasm at the lower pole of the nucleus; such polar bodies are often seen in the affected cells. Intracytoplasmic inclusions are seen as dark particles below and to the right, adjacent to the lumen of the acinus, which in this tangential section is just visible below the affected cell. Haematoxylin-eosin. ×850.

proportion to the enlargement of the nucleus and the nucleocytoplasmic ratio remains about normal. The mature intranuclear inclusion bodies consist mainly of deoxyribonucleic acid, with some ribonucleic acid (Seifert, 1956; Symmers and Humberstone, 1959): they are probably formed mainly of virus particles.

The intracytoplasmic inclusions in the cells of salivary duct epithelium accumulate as rather coarsely granular masses between the nucleus and the free margin of the cell, causing the cell to bulge into the lumen. They consist largely of neutral mucopolysaccharides and are probably secretory substances which have accumulated because of interference with nuclear function. Traces of deoxyribonucleic acid and ribonucleic acid are usually present also, and larger amounts may appear when the ageing intranuclear inclusion body begins to lose its concentration of these substances during the period before the degenerating cell is shed into the lumen (Seifert, 1956; Symmers and Humberstone, 1959).

Extent of the Infection in the Body

(a) Localized Cytomegalic Inclusion-body Disease in Infancy.—In the great majority of cases the disease in infants is apparently confined to the salivary glands, particularly the serous glands. The epithelium of the ductules is affected almost exclusively, inclusion bodies being rarely found in acinar cells.

(b) Localized Cytomegalic Inclusion-body Disease in the Adult.—It is usually said that the salivary glands are not affected in adults, and in fact their involvement has only once been recorded (Symmers, 1960). It is debatable whether any localized form of cytomegalic inclusion-body disease occurs in adults which is at all comparable with the symptomless and clinically unimportant localized infection in infants. While cases have been recorded in which the infection was observed only in one part of the body, the circumstances have always been very different from those in infancy; the cytomegaly has usually been in the vicinity of a definite lesion, such as ulceration of part of the alimentary tract (five cases), or it has been confined to the lungs (six cases), when it is usually associated with pneumocystis infection or some other form of pneumonia complicating Hodgkin's disease or another serious illness.

(c) Generalized Cytomegalic Inclusion-body Disease in Infancy.—The organs involved in generalized infections in infancy are, in order of frequency, salivary glands, kidneys, and lungs, then liver and pancreas, and then thyroid, adrenals, gastrointestinal tract, and brain. Other organs and tissues are rarely affected.

(d) Generalized Cytomegalic Inclusion-body Disease in the Adult.—In adults the relative liability of the various organs to the infection differs somewhat from that in infancy; those most heavily involved have been the lungs, adrenals, spleen, intestine, and kidneys, approximately in that order.

The virus is not exclusively epitheliotropic, and in generalized infections vascular endothelium, mesothelium, fibroblasts, and macrophages, and occasionally muscle cells, may be infected: this seems particularly likely to be the case in adults.

Other Changes in Infected Organs.—Heavily affected organs show focal interstitial inflammation, with a predominantly lymphocytic and plasma-cell infiltrate. Focal necrosis is common in the adrenals and liver when they are heavily involved.

Incidence

The average incidence of cytomegalic inclusion-body disease in infants and children, as shown by regular histological examination of salivary glands, was between 10 and 15% of some 1,700 necropsies in various parts of the world (China, Czechoslovakia, East Indies, Germany, Hungary, United States of America, and Venezuela) between 1932 and 1956 (Seifert and Oehme, 1957). Generalized infection is considerably less frequent: the studies which Seifert and Oehme (1957) reviewed indicated an incidence of between 1 and 3% of necropsies in childhood. My impression from discussions with colleagues who see considerably more material from infants and young children than I do is that both localized and generalized infections are less frequent in Britain than in the countries where the figures noted above were obtained.

The adult type of the disease, which is met with in older children as well as in adults, is undoubtedly very rare. Details of the published cases have been tabulated by Seifert and Oehme (1957), Geiler (1957),* and Fisher and Davis (1958). To the 25 cases collected in these three reviews may be added a case reported by Takos (1956), the case of Sakamoto and Oota (1957), three cases reported by Peace (1958) and the three cases in this paper, as well as a case of involvement of a parotid gland (Symmers, 1960). Nineteen of

*Geiler's (1957) case of a 7-year-old boy with the adult type of cytomegalic inclusion-body disease was also included by Seifert and Oehme (1957) as case 83 of their series.

these 34 cases were examples of inclusion-body cytomegaly localized to one organ, and 15 were examples of generalized infection; in some of the cases in which only one organ was found to be involved the specimen was obtained surgically and the true extent of the infection in the body is therefore unknown.

Association with Pneumocystis Pneumonia

Both in infants and in adults cytomegalic inclusion-body disease has been associated notably often with another unusual infection, pneumonia caused by *Pneumocystis carinii*. This organism is usually described as a protozoon but its nature is in fact uncertain, and it is thought by some workers to be a fungus; it has not yet been cultivated. Pneumocystis infection has been made familiar to us by Baar (1955) and Hamperl (1957); it is rarer in this country than in continental Europe. The alveoli of affected parts of the lungs are filled by the characteristic colonies of the organism. The pneumonic reaction is practically confined to the interstitial tissue (Fig. 3), and plasma cells are so conspicuous that the name interstitial plasma-cell pneumonia is peculiarly apt, although not every case of pneumonia for which this name would be appropriate is due to pneumocystis infection; other causes are said to include cytomegalic inclusion-body disease itself.

Pneumocystis pneumonia was present in 35 of the 78 cases of cytomegalic inclusion-body disease in children under 1 year of age studied personally by Seifert and Oehme (1957). Sixteen of their 78 cases were cases of generalized cytomegalic inclusion-body disease, and no less than 10 of these 16 showed pneumocystis infection. Similarly, pneumocystis infection, which ordinarily is very rare indeed in adults, was recognized with certainty in six of the 15 recorded cases of the adult type of generalized cytomegalic inclusion-body disease, including all three of the cases reported in this paper; it was probably present in at least four more of the published cases.

The coexistence of these two unusual infections is too frequent to be due to chance. Possibly there is some biological synergism between *Pneumocystis carinii* and the virus of cytomegalic inclusion-body disease. On the other hand, it is possible that some independent circumstance or combination of circumstances predisposes to both infections; resistance to them may be low in infants because of prematurity and in adults because of some other illness or of an effect of the drugs with which it has been treated.

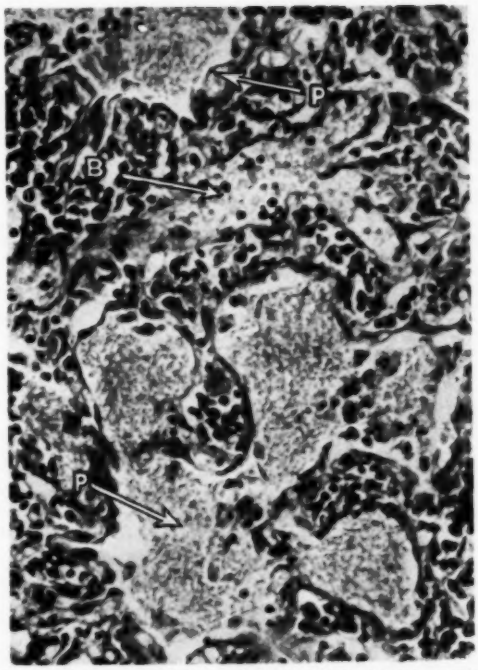

FIG. 3.—Case 1: Typical picture of plasma-cell interstitial pneumonia with intra-alveolar pneumocystosis. The appearances are indistinguishable from those seen in pneumocystis pneumonia in infancy. P—colonies of *Pneumocystis carinii* filling alveoli. B—blood vessel. Haematoxylin-eosin. ×350.

Predisposing Conditions

It is debatable whether prematurity predisposes to the infantile type of cytomegalic inclusion-body disease or is caused by it. Haemolytic disease of the newborn has also been said to predispose to this infection, but again the evidence seems to be more in favour of the infection being one of the rarer causes of that syndrome (Cappell and McFarlane, 1947). In contrast, there is no doubt that predisposing diseases play an important part in the aetiology of the adult type of cytomegalic inclusion-body disease, particularly the generalized form. An apparent predisposing cause was present in 12 of the 15 cases of the generalized infection in adults and in six of the 20 other adult cases on record. The associated condition was a primary malignant disease of the lymphoreticular system (Hodgkin's disease or lymphosarcoma) or leukaemia in 10 cases and aplastic anaemia in two cases; paraplegia, hypertensive nephrosclerosis, glomerulonephritis, ulcerative colitis, Wegener's granulomatosis, and thrombotic purpura accounted

for the other six cases. The last two of these cases and one of the cases of Hodgkin's disease are reported in this paper.

Case 1: Generalized Cytomegalic Inclusion-body Disease, Pneumocystis Pneumonia, and Wegener's Granulomatosis

A 34-year-old American schoolmistress was troubled slightly by deafness in the right ear in May and again in November, 1956. In May, 1957, she was given an unidentified antibiotic by mouth because of throbbing tinnitus in the left ear. Her general health had always been good. While on her way from America to Britain she had coryzal symptoms followed by a persistent blood-streaked discharge from the nose during an outbreak of influenza in the ship, and on the day of arrival here (July 8, 1957) her left ear became painful and deaf. Two days later she called at a hospital: acute suppurative otitis media was found, and she was given an injection of penicillin. She did not attend for the rest of the prescribed course of injections because she was anxious not to interrupt a study tour. On July 15 she again went to a hospital: she attended for four daily injections of penicillin, and on alternate days chloramphenicol was insufflated into the ear, from which pus discharged freely. After this she decided to continue her journey.

On July 23 she arrived in London and was obliged by severe pain to go straight into hospital with acute mastoiditis. Temperature 102.2° F. (39° C.). Leucocyte count 12,000 per c.mm.; haemoglobin 11.5 g. per 100 ml. Urine showed a trace of protein, and there were a few erythrocytes in the centrifugate.

Simple mastoidectomy was performed on July 24: the mastoid air cells were acutely inflamed and contained sterile mucopus; the granulation tissue removed from the mastoid was sent for histological examination (see below). The lateral sinus was not involved, and needling showed that it contained normal blood.

Her general condition did not improve after the operation: although the aural symptoms were promptly relieved she continued to feel and look very ill. During the following week her temperature ranged between 100 and 103° F. (37.8 and 39.4° C.), with proportionate tachycardia. Her respirations rose to 42 a minute. She coughed a great deal, producing purulent sputum, cultures of which yielded heavy growths of *Escherichia coli* but nothing else of note. In spite of treatment with antibiotics (see Table) and probenecid ("benemid") there was no sustained improvement. Radiographs showed increasingly widespread, patchy consolidation in both lungs, and by August 7 some of these lesions were beginning to show cavitation. The likeliest diagnosis at this time was thought to be multiple lung abscesses due to embolism, secondary to septic thrombosis of the lateral sinus as a complication of the mastoid infection, although by then the mastoid wound had healed and the ear itself was dry. Repeated blood

TABLE I
ANTIBIOTIC AND STEROID THERAPY IN CASE 1

Drug	Length of Course	Administration	Total Dosage
Oxytetracycline (terramycin)	i, July 23–26	0·25 g. by mouth 6-hourly (11 doses)	2·75 g.
	ii, July 30–31	0·5 g. by mouth 6-hourly (4 doses)	2·0 g.
Streptomycin ..	i, July 26–29	1·0 g. intramuscularly twice daily (8 doses)	8·0 g.
	ii, July 30–Aug. 3	Ditto (9 doses)	9·0 g.
	iii, Aug. 9–13	,, (8 ,,)	8·0 g.
Benzylpenicillin*	i, July 27–29	2,500,000 units intramuscularly 4-hourly (14 doses)	35,000,000 units
	ii, Aug. 9–13	2,000,000 units intramuscularly thrice daily (12 doses)	24,000,000 units
Erythromycin	July 31–Aug. 7	0·5 g. by mouth thrice daily (20 doses)	10·0 g.
Tetracycline (achromycin)	Sept. 5–10	0·5 g. by mouth 6-hourly (20 doses)	10·0 g.
Prednisolone ..	Aug. 26–Sept. 16	10 mg. by mouth 6-hourly (84 doses)	840 mg.

* The patient had also had an injection of penicillin daily from July 15 to 18 inclusive. The dose and preparation are not known. (The left ear was insufflated with chloramphenicol twice during the same period; chloramphenicol was not administered internally.)

cultures were sterile. The urine contained many erythrocytes and occasional granular casts; protein ++. Neutrophil leucocytosis persisted, and the haemoglobin fell to 9.2 g. per 100 ml. Blood transfusions were given, and during one of these (August 3) she developed acute, severe, generalized urticaria, which responded promptly to the intramuscular injection of 50 mg. of promethazine hydrochloride ("phenergan"). After this episode promethazine was given by mouth for several days, with supplementary parenteral administration during and after blood transfusions: no further urticaria developed.

The right kidney was explored surgically on August 10 because of tenderness and fullness in the lumbar region, first noticed a week earlier. The tissues round the kidney were healthy, but the kidney itself was swollen and there was bloody fluid beneath its capsule, which was therefore stripped. There was a febrile reaction (101.2° F. [38.4° C.]; pulse 130) during a blood transfusion after this operation: intense generalized flushing accompanied the reaction but there was no shock, pain, or rigor; the transfusion was completed at a reduced rate and nothing further happened until some hours later, when the urine was found to be heavily blood-stained.

Haematuria persisted, punctate erythema developed and became confluent, and there were epistaxes. Bedsores formed rapidly where the rash had been heaviest over the buttocks. The blood urea level began to rise; acidosis, sodium deficiency, and increasing fluid retention developed, and attempts to maintain fluid and electrolyte balance were only partly successful. Oedema, congestive heart failure, and progressive uraemia were accompanied by persistent

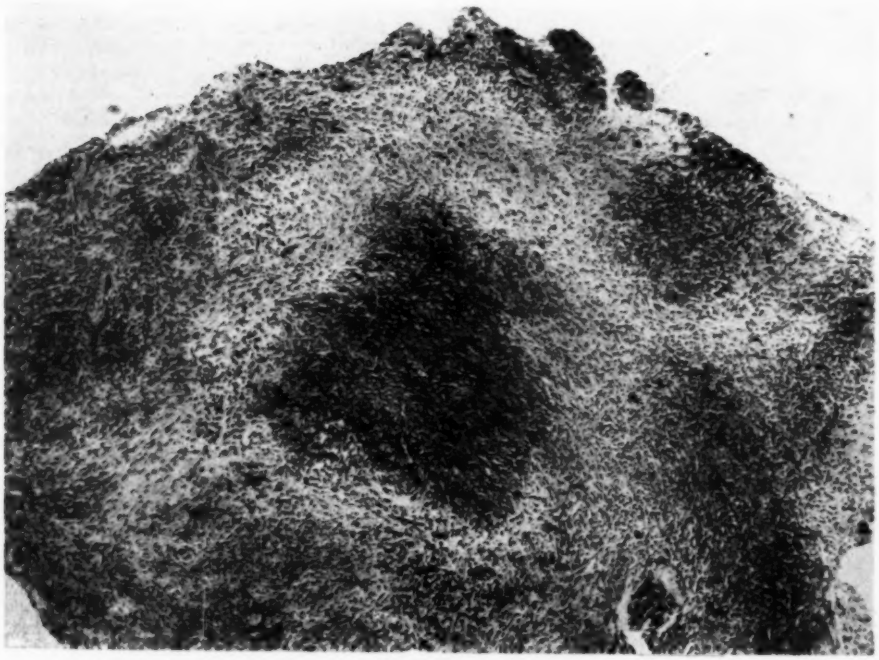

FIG. 4

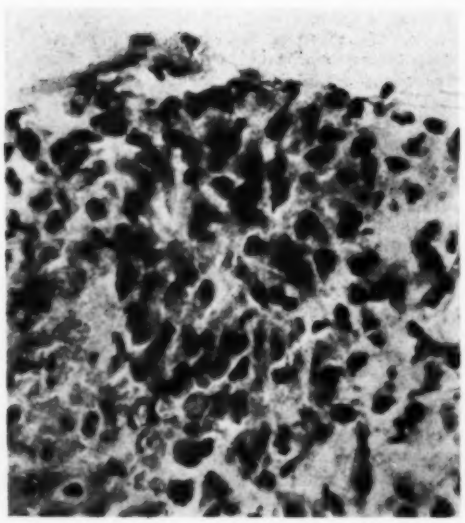

FIG. 5

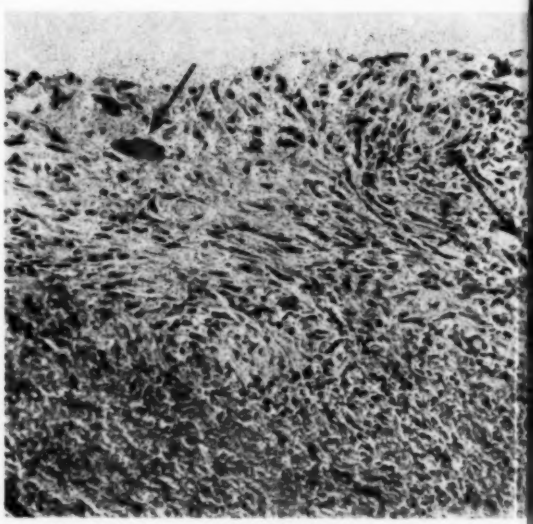

FIG. 6

FIG. 4.—Case 1: Mastoidectomy specimen. Low-power view showing extensive foci of necrosis (darker areas) and intervening fibrosis. Haematoxylin-eosin. ×45.

FIG. 5.—Case 1. Mastoidectomy specimen. "*Radiärknötchen*," focal collection of more or less radially orientated cells. Haematoxylin-eosin. ×600.

FIG. 6.—Case 1: Mastoidectomy specimen. Multinucleated giant cells (arrowed) in organizing granulation tissue adjoining a necrotic focus (below). Haematoxylin-eosin. ×90.

FIG. 7.—Case 1: Lung. Typical multinucleated giant cells of Wegener's granulomatosis, with hyperchromatic, compact nuclei. Haematoxylin-eosin. ×300.

FIG. 8.—Case 1: Mastoidectomy specimen. Inclusion-body cytomegaly. Haematoxylin-eosin. ×700.

FIG. 9.—Case 1: Lung. Pulmonary arteritis, showing palisading of cells around necrotic media, and thrombosis. Haematoxylin-eosin. ×220.

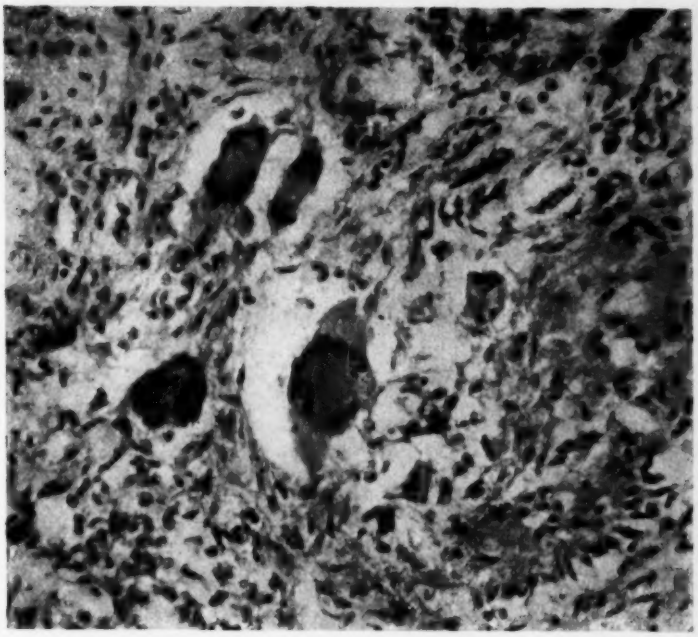

FIG. 7

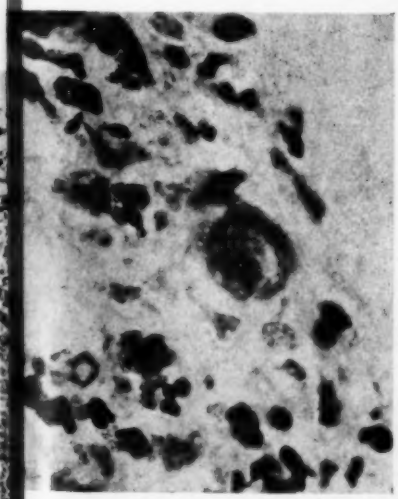

FIG. 8

FIG 9

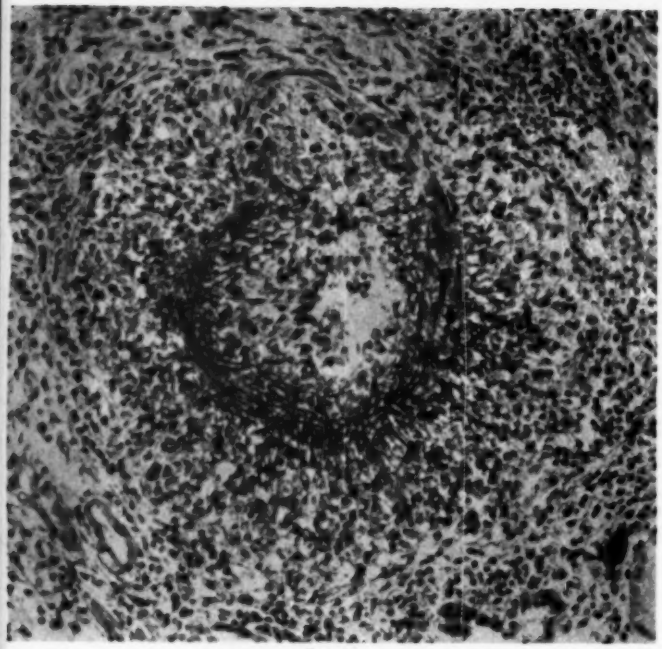

FIG. 10

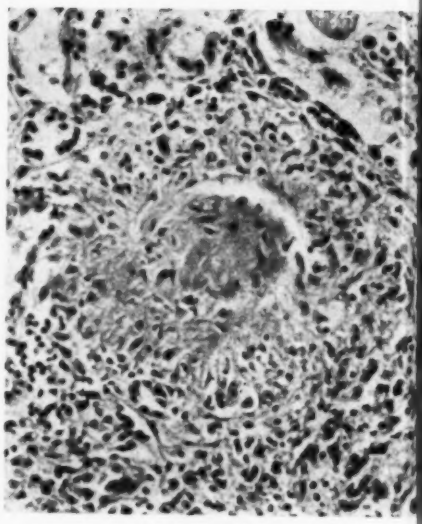

FIG. 12

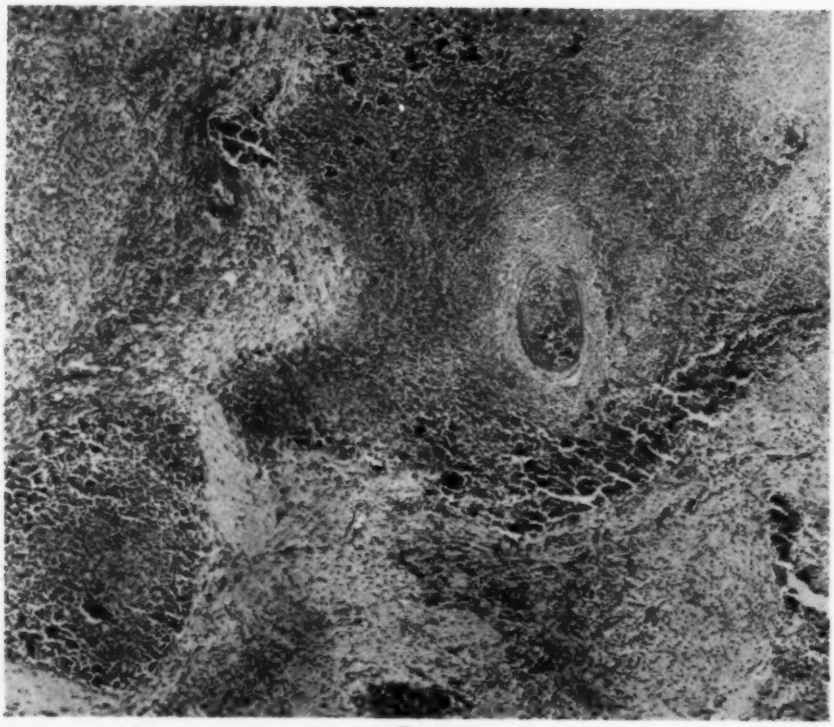

FIG. 11

FIG. 10.—Case 1: Kidney. Acute segmental arteritis. Haematoxylin-eosin. ×200.

FIG. 11.—Case 1: Spleen. "*Fleckmilz.*" infarction due to arteritis; the extensive dark stippling of the dead tissue is due to calcification. Haematoxylin-eosin. ×40.

FIG. 12.—Case 1: Kidney. Granulomatous nephritis of Wegener's disease. Necrosis of the glomerulus with radiating circumglomerular granuloma. Haematoxylin-eosin. ×220.

FIG. 13.—Case 1: Fibrinoid necrosis of part of a glomerulus. Haematoxylin-eosin. ×600.

FIG. 14.—Case 1: Lung. Interstitial fibrosis with preservation of alveolar pattern, conspicuous alveolar cell lining, and intra-alveolar collections of desquamated cells. The picture is that seen in certain cases of the Hamman-Rich syndrome. High magnification showed no evidence of inclusion-body cytomegaly in such fields as this, in contrast to areas such as that shown in Fig. 15. Haematoxylin-eosin. ×100.

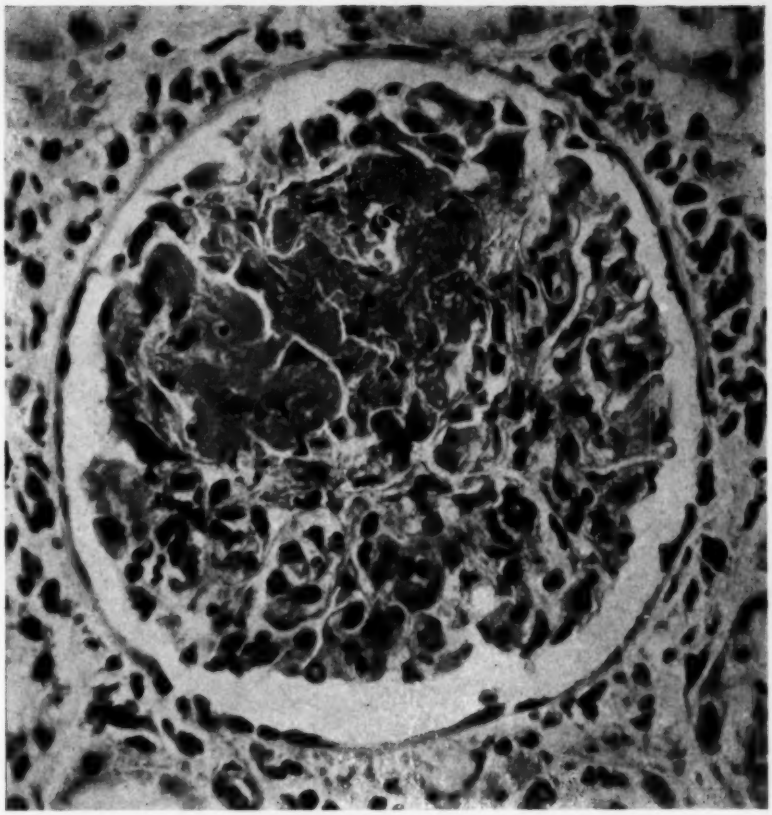

FIG. 13

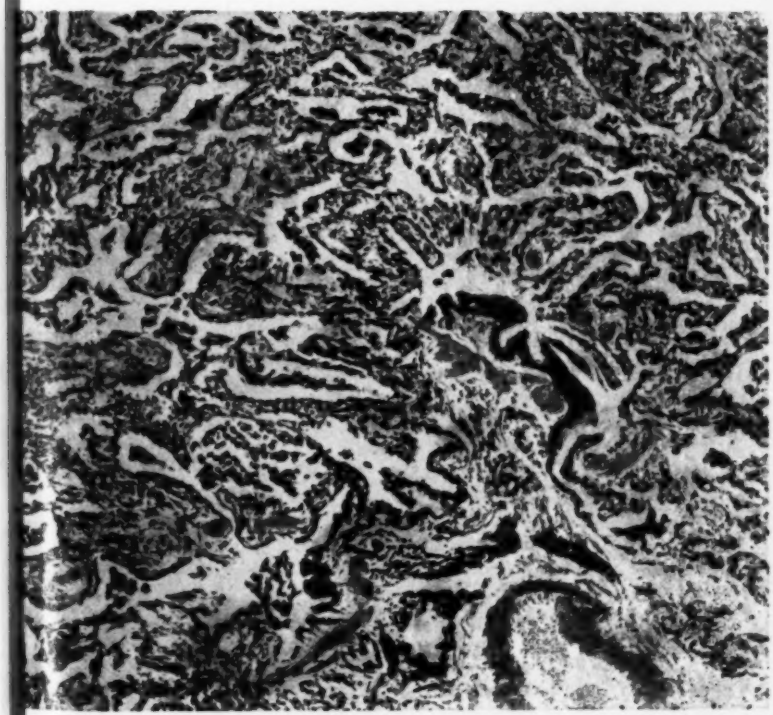

FIG. 14

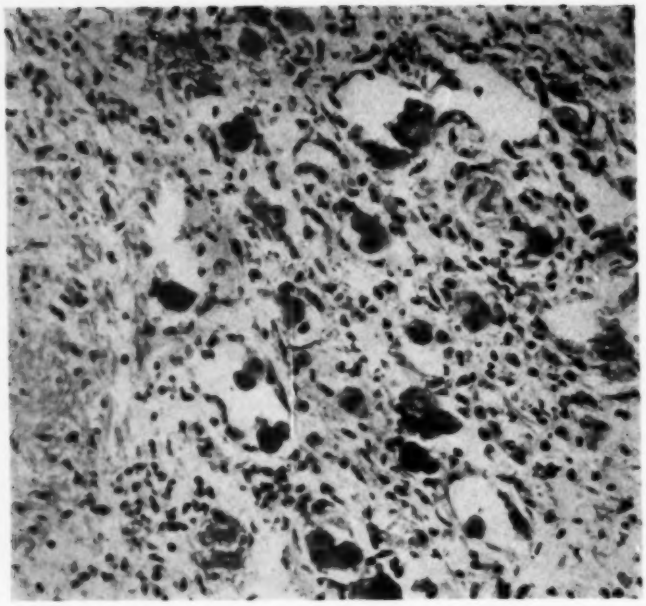

FIG. 15

FIG. 15.—Case 1: Lung. An area of interstitial fibrosis with inclusion-body cytomegaly in the alveoli. The adjacent tissue includes a necrotic focus of Wegener's granulomatosis, with a characteristic multinucleated giant cell. Haematoxylin-eosin. ×220.

FIG. 16.—Case 1: Colonic ulcer: inclusion-body cytomegaly. Extensive infiltration of muscle coat and subserosa by collections of affected cells. At C endothelium of a blood vessel is heavily involved. (A, Auerbach's plexus [normal].) Haematoxylin-eosin. ×55.

FIG. 17.—Case 1: Higher magnification of part of field shown in Fig. 16. ×220.

FIG. 18.—Case 1: Inclusion-body cytomegaly of adrenal cortex. Haematoxylin-eosin. ×200.

FIG. 19.—Case 1: Pneumocystis infection. The lace-like appearance is typical of a colony of *Pneumocystis carinii*. The field is from a focus of terminal suppurative bronchopneumonia quite unrelated to the presence of pneumocystosis. Silver preparation for reticulin; haematoxylin counterstain. ×300.

FIG. 20.—Case 1: Field corresponding to that in Fig. 19, showing colonial appearance more clearly in a periodic-acid-Schiff preparation (Mayer's haemalum counterstain). ×300.

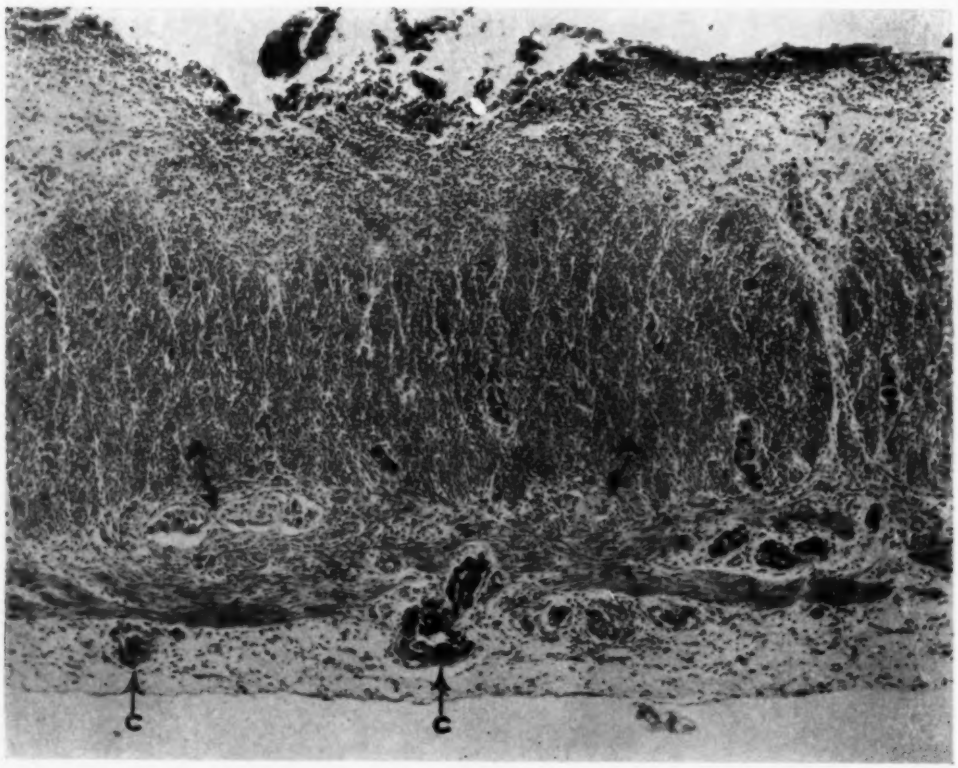

FIG. 16

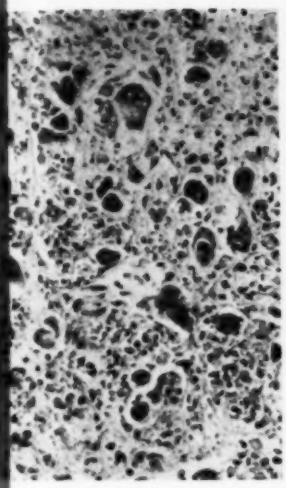

FIG. 18

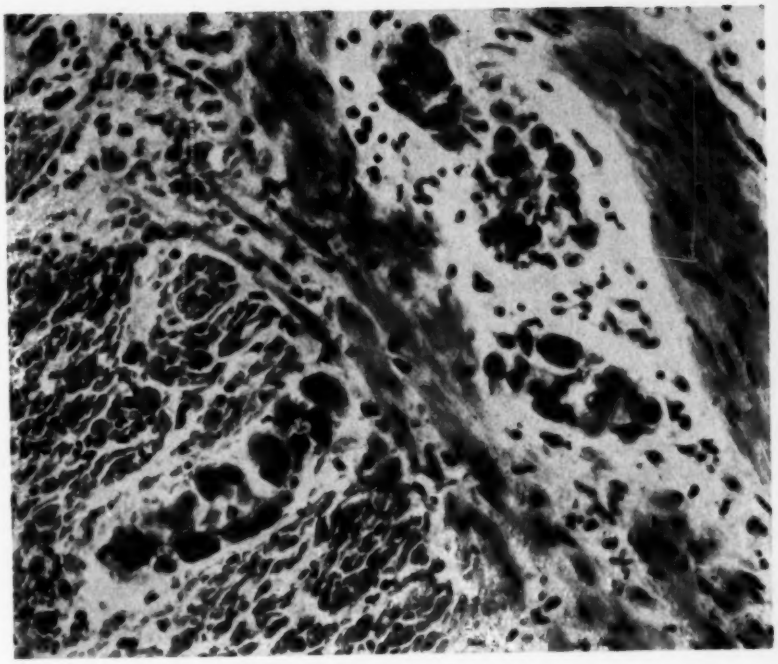

FIG. 17

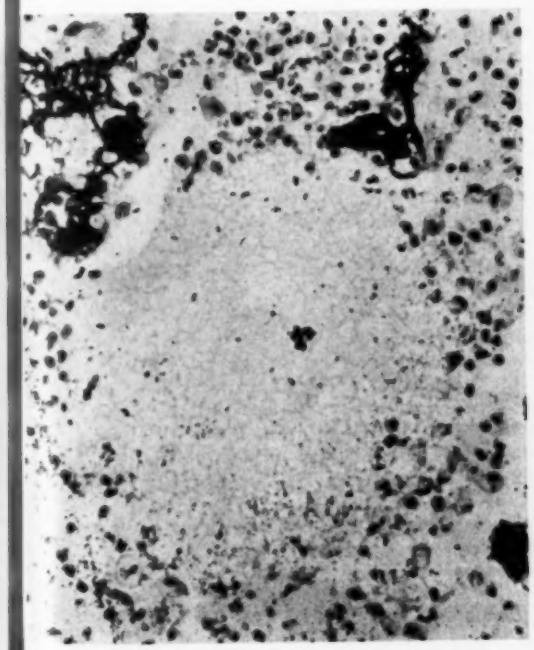

FIG. 19

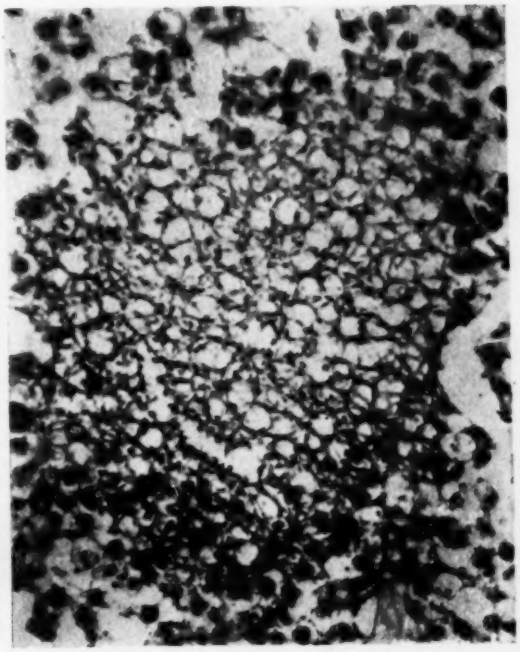

FIG. 20

haemorrhagic manifestations and progressive anaemia in spite of transfusions. Crops of purpura began to appear, without thrombocytopenia.

L.E. cells were looked for on one occasion only: none was found.

It was now (August 26) considered that Wegener's granulomatosis was the diagnosis likeliest to account for the complex clinical picture. Treatment with prednisolone was started (see Table), and continued until the patient's death in uraemic coma on September 16. There was some passing improvement in the first week of this course, the fever and tachycardia disappearing, but the biochemical disturbances became severer and could not be controlled. Her blood pressure was normal until the last week of the illness, when it ranged about 170/100 mm. of mercury. The chest lesions had begun to resolve shortly before the course of prednisolone was begun, and this improvement continued until the week of her death. Purpura recurred three days before her death, but only on the limbs. On September 13 haemoglobin was 7.7 g. per 100 ml.; erythrocytes 2,330,000 per c.mm.; leucocytes 17,000 (98% neutrophils); platelets 38,000 per c.mm.

Necropsy Findings.—The necropsy was performed 22 hours after death. There was considerable purpura on the limbs.

Respiratory System.—The mastoidectomy site had healed. The nasal passages, nasopharynx, larynx, and trachea were healthy. There was suppurative bronchopneumonia in both lungs and some patchy hyperaemia and carnification. Several ill-defined, abscess-like lesions, none more than 2 cm. in diameter, were present in both lungs, accounting for the confluent shadows in the radiographs. Filmy pleural adhesions were present but no active pleurisy.

Cardiovascular System. — Generalized fibrinous pericarditis was present. The myocardium was soft but the heart was otherwise healthy. No thrombosis was found in the heart or elsewhere.

Central Nervous System.—The dural venous sinuses, meninges, and brain were healthy.

Alimentary System.—The only abnormalities were in the liver and colon. The liver (2,365 g.) was pale and appeared to be somewhat fatty; two encapsulated, whitish, ovoid structures (3 by 1 cm., and 2 by 1 cm.) were present within the left lobe, apparently in continuity with portal tracts. The colon near the splenic flexure was intensely hyperaemic throughout an annular zone which contained a few shallow ulcers, the largest of which measured 0.75 by 0.25 cm.; the larger dimension of the ulcers was in the long axis of the bowel.

Lymphoreticular System.—The spleen (225 g.) showed almost complete infarct-like necrosis, only a narrow subcapsular and circumvascular zone appearing to have survived. The splenic artery and vein were healthy. There was no lymphadenopathy.

Urinary System.—The kidneys (left 280 g., right 240 g.) were swollen; the cortex was pale and contained several anaemic infarcts, none of which was more than 0.25 cm. in its longest dimension. The urinary passages and bladder were normal.

Endocrine System.—The adrenals were pale and soft; the other glands appeared normal. The reproductive organs were healthy.

Histological Studies*

Mastoidectomy Specimen.—The tissue removed during the mastoidectomy eight weeks before death was examined histologically at that time and reported upon as "simple granulation tissue, partly necrotic." It is unfortunate that more attention was not given to the interpretation of this specimen, from which the diagnosis of both Wegener's granulomatosis and cytomegalic inclusion-body disease should have been made. It was originally overlooked that the tissue showed considerably more organization and fibrosis than was consistent with the comparatively short duration (16 days) of acute otitis before the operation. Moreover, there were many necrotic foci which together occupied a considerable proportion of the tissue (Fig. 4): the abundance of nuclear debris in these foci, the unusually sharp demarcation of much of the necrotic matter from the surrounding fibrotic tissue, and the suggestion in places of peripheral histiocytic palisading should have indicated that the appearances were not consistent with a banal suppurative process. In addition, in the more actively inflamed parts there were occasional radiate nodules (Fig. 5), like the *Radiärknötchen* described by Miescher (1942) in erythema nodosum (Reich, 1952). Although these nodules are not specific, recognition of their presence is helpful because they indicate the possibility of an allergic or other unusual reaction. Eosinophils were present but very scanty.

The most important diagnostic feature of this biopsy was the presence of multinucleated giant cells of the type which are so characteristic of Wegener's granulomatosis that when found in necrotic tissue in any part of the respiratory tract they are virtually pathognomonic (Figs. 6 and 7). These cells appear at first sight to be similar to the typical Langhans giant cell of tuberculosis: their nuclei, however, are smaller and more compact than those of the Langhans cell, and are hyperchromatic, even pyknotic, in appearance or, alternatively, show the curious muddy, greyish-lilac colour in haematoxylin-eosin preparations which commonly indicates depolymerization of deoxyribonucleic acid. The nuclei are usually

*All the tissues studied in these three cases were fixed in formol-saline (4% formaldehyde in normal saline).

densely clustered, sometimes in radial formation, at one or both poles of the cell, or form a group at its centre. The cytoplasm of the Wegener cell is more eosinophil and more hyaline than that of the giant cell of tuberculosis.

When cytomegalic inclusion-body disease was recognized in the post-mortem material, the 20 available serial sections of the biopsy specimen were reviewed: two vascular endothelial cells were found which showed the characteristic nuclear changes (Fig. 8).

Post-mortem Material.—Histological examination confirmed the clinical diagnosis of Wegener's granulomatosis and also showed the presence of generalized cytomegalic inclusion-body disease and of pneumocystis pneumonia. The findings relevant to these conditions may most conveniently be dealt with under separate headings, with additional notes on some observations which do not clearly fall into these categories.

Wegener's Granulomatosis.—The pathological criteria for the diagnosis of Wegener's granulomatosis are (1) necrotizing giant-cell granulomatosis of the respiratory tract, with involvement both of the upper air passages (or their adnexa) and of the lungs, (2) necrotizing angeitis, and (3) glomerulonephritis. These criteria were amply fulfilled in this case.

(1) The mastoid lesion represented the characteristic upper respiratory tract involvement. In the lungs necrotizing pneumonia typical of Wegener's disease accounted for the macroscopical abscess-like lesions. Fibrosis of some of the pulmonary lesions had converted considerable parts of the affected tissues into scars, still heavily infiltrated by inflammatory cells, with fresh necrotic foci sparsely scattered through the tissue. Tubercle-like formations were seen but were infrequent. Occasionally, a striking focal proliferation of smooth-muscle cells was found in the Wegener lesions, and characteristic Wegener giant cells in these foci sometimes appeared to have developed in such intimate association with muscle cells that their origin from muscle cells seemed possible. Implication of smooth muscle was seen in another form in the necrosis of the media of pulmonary arteries (Fig. 9); marginal histiocytic palisading was also a feature of the arteritic changes. Some of these arteries were filled by completely organized and partly recanalized granulation tissue, presumably following thrombotic occlusion, and their walls were sometimes breached by segmental fibrous scars. The arterial lesions were confined to the foci of diffuse pulmonary fibrosis, and it is therefore possible that some of these were organized infarcts.

(2) Angeitis was found in skeletal muscle, kidneys, pancreas, lungs, and spleen: small arteries and arterioles were predominantly involved, but occasionally also small veins. Some of the lesions were healed or healing; rarely, others, particularly in the kidneys, were in an acute phase (Fig. 10) and indistinguishable from the lesions of acute polyarteritis. Eosinophils were present, but very sparse, in the exudate in the acute lesions. The appearances in the spleen were those of polyarteritis, with infarction, trabeculitis, and folliculitis; parts of the necrotic tissue were heavily calcified (Fig. 11). The two encapsulated lesions in the liver were thrombosed and organizing polyarteritic aneurysms.

(3) Glomerulitis was conspicuous, most of the glomeruli being involved. The lesions were of different ages; some were healing and others were fresh, either with necrosis or with conspicuous granuloma formation involving the glomerulus and the immediately surounding tissue (Fig. 12).

Findings Reminiscent of Systemic Lupus Erythematosus.—Some glomeruli showed changes which were more typical of systemic lupus erythematosus than of Wegener's disease. These ranged from segmental thrombosis and segmental fibrinoid necrosis (Fig. 13) to the typical "wire-loop" change in the capillary basement membrane. Further, in some of the necrotic foci, for example, in the spleen and in the Wegener lesions in the lungs, haematoxyphil bodies like those of systemic lupus erythematosus were readily found. Protracted search of such areas showed occasional unmistakable instances of phagocytosis of altered nuclei by neutrophil polymorphonuclear leucocytes, an observation which is possibly the histological equivalent of the L.E. phenomenon (Symmers, 1958).

Interstitial Pulmonary Fibrosis (Hamman-Rich Type).—In parts of the lungs there were foci of purely interstitial fibrosis, and the alveolar pattern was preserved: the alveoli in these foci contained aggregates of large mononuclear cells (Fig. 14). The picture was indistinguishable from that which may be seen in advanced stages of progressive interstitial pulmonary fibrosis in the Hamman-Rich syndrome (Hamman and Rich, 1944; Peabody, Buechner, and Anderson, 1953). These foci could not be clearly related either to the lesions of Wegener's granulomatosis or to the interstitial pneumonia associated with infection by *Pneumocystis carinii* (see below), but the latter would be a likelier origin in view of the destructive

nature of the Wegener lesions. In infancy both cytomegalic inclusion-body disease and pneumocystis infection may cause interstitial pneumonia and eventually interstitial pulmonary fibrosis; in the present case the cytomegalic infection was very much more widespread than the interstitial fibrosis, and although it was more prominent in areas of fibrosis than elsewhere in the lungs (Fig. 15) there were fibrotic foci which were free from cytomegaly (Fig. 14).

Cytomegalic Inclusion-body Disease. — Inclusion-body cytomegaly was present in every tissue examined. The heaviest involvement was in the colonic ulcers, lungs, spleen, adrenals, and skin. The colonic ulcers, in the absence of any other apparent local cause, such as arteritis, may have been due to the viral infection; there were many conspicuous accumulations of affected cells, probably histiocytes, in the mucosa and submucosa forming the edges and floor of the ulcers, and aggregates of these cells were present in the muscle coats, following the lymphatic pathways, and in the subserosa. The appearances at low magnification were remarkably like those of carcinomatous infiltration (Fig. 16), but higher magnification immediately showed their real nature (Fig. 17). Vascular endothelial cells were conspicuously involved in the vicinity of the colonic lesions (Fig. 16), and mesothelial cells were occasionally affected. Throughout the affected tissues in all parts of the body, however, involvement of free-lying cells in the interstitial tissues, presumably histiocytes, predominated.

Epithelium was relatively seldom affected, except in the adrenals; occasional cells of the kidney (mainly proximal convoluted tubules), pancreas (ducts and islet cells), and bronchial glands (Fig. 2) were involved. In the adrenals there were many foci of heavy involvement of the cortical cells (Fig. 18), and there was extensive central necrosis in the larger lesions: the shadow cells in these necrotic foci still showed clear traces of cytomegalic changes, and any possibility that the infection was secondary to the death of the tissue seemed to be excluded.

In the lungs inclusion-body cytomegaly was found mainly in two situations, in ulcers of the bronchi and in the alveoli (Fig. 15). There was nothing to suggest that the bronchial ulcers had occurred as part of the Wegener's disease; their appearances were comparable with the cytomegalic ulcers in the colon.

In the spleen it was the cells lining the sinusoids and occasional free pulp cells which were involved. In the infarcted parts of the spleen inclusion-body cytomegaly was still recognizable among the shadow cells, and in the calcified parts all stages of calcification of the infected cells could be seen.

In the skin involvement was heaviest in purpuric areas, and capillary endothelium was predominantly infected. There were no other lesions in the skin.

The findings strongly suggested that the colonic and bronchial ulceration, the cutaneous purpura, and much of the focal adrenal cortical necrosis were due directly to cytomegalic inclusion-body disease.

Adrenal Cortical Necrosis.—As well as the necrosis of cytomegalic foci there were many smaller foci of necrosis in which no evidence of infection was to be seen: these foci were of the cytolytic type such as is seen after treatment with cortisone and its analogues.

Parenchymatous Cytocalcinosis. — Calcification of parenchymatous cells, sometimes in foci of necrosis and sometimes without other apparent alteration in the affected field, was present in small areas of myocardium, kidneys (tubular epithelium), liver, adrenal cortex, anterior lobe of the pituitary, and thyroid. It seems probable that this, and doubtless also the widespread calcification of the necrotic splenic tissue, resulted from the disturbances in acid-base balance which were so troublesome clinically.

Pulmonary Infection by Pneumocystis carinii. —When it was found that cytomegalic inclusion-body disease was present the available sections of lung were searched for evidence of pneumocystis infection, in view of the known association of the two infections. At first nothing to suggest pneumocystis was found in the haematoxylin-eosin preparations of the eight blocks of lung tissue available. However, when examining the reticulin pattern in some silvered preparations in which a light haematoxylin nuclear stain had been used a single, clear, rounded area was noticed in a bronchopneumonic area (Fig. 19); at first this was thought to be an artefact due to the nuclear stain failing to take, and no particular attention was paid to it. Later, while looking at a periodic-acid-Schiff preparation of an adjacent section from the same block it was seen that the corresponding field was in fact occupied by a network of Schiff-positive material enclosing minute rounded spaces (Fig. 20). This so closely resembled a colony of pneumocystis that further blocks of lung tissue were prepared: two of these contained small areas of typical plasma-cell interstitial pneumonia with alveolar pneumocystosis (Fig. 3).

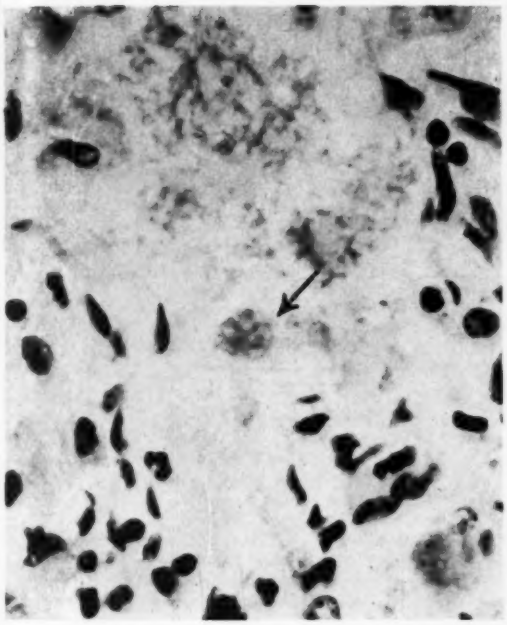

FIG. 21.—Case 2: Pneumocystis infection. An alveolus filled with the organisms, of which only the arrowed cluster and occasional single parasites have shown at all well in spite of the periodic-acid-Schiff reaction (Mayer's haemalum counterstain). ×600.

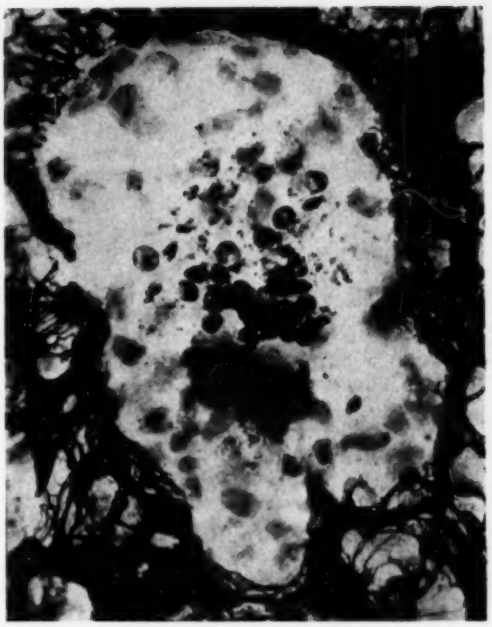

FIG. 22.—Case 2: Field corresponding to that in Fig. 21, showing individual organisms clearly (and some of their internal structure). Silver preparation by Mr. F. D. Humberstone (1958). ×600.

Although the appearance of the colonies of *Pneumocystis carinii* is distinctive and practically unmistakable in haematoxylin-eosin and periodic-acid-Schiff preparations (Fig. 3) the individual organisms are not readily demonstrable by these methods (Fig. 21); they can be beautifully shown by silver methods as used by Le Tan (1954), and particularly in a modification developed by F. D. Humberstone (1958) in my laboratory for use on paraffin sections of formol-fixed material (Fig. 22). The Gomori methenamine (hexamine) silver-nitrate method as modified by Grocott (1955) also gives a very clear and delicate picture of these organisms.

Commentary on Case 1

Steroids have been temporarily helpful in some cases of Wegener's granulomatosis, and sometimes have induced remissions of considerable duration (Fahey, Leonard, Churg, and Godman, 1954). Plummer, Angel, Shaw, and Hinson (1957a) stressed the desirability of beginning to give steroids before the onset of renal failure. Steroid therapy was begun late in the course of the case reported here, which illustrated the characteristically unremitting progress of the renal involvement in spite of considerable healing of the pulmonary and arteritic lesions and complete healing of the disease in the mastoid. How far treatment contributed to healing is debatable, and it is not clear why the renal changes did not react correspondingly well: at least it may be said that the histological findings are an encouragement to continue to try the effect of steroids in future cases of Wegener's disease, in spite of the attendant risks.

Wegener's granulomatosis is not to be confused with the so-called lethal midline granuloma of the face (" granuloma grangraenescens ") (see Resnick and Skerrett, 1959). Its kinship to polyarteritis, and, more remotely, to systemic lupus erythematosus, places it within the heterogeneous group of diseases primarily affecting the connective tissues (the so-called " collagen diseases "). The finding in the case under discussion of histological features usually associated with systemic lupus erythematosus (haematoxyphil bodies; phagocytosis of altered nuclei; glomerular changes) reflects the overlap of the pathogenic

manifestations of the two entities, but is not evidence of any particularly close relationship between them.

This case, like others which have been reported, illustrated the proneness of patients with Wegener's granulomatosis to show unusual sensitivity reactions, in this case urticarial and febrile reactions during blood transfusions. The erythematous skin rash was also, for a time, thought to be due to sensitivity (to penicillin), but in fact it may have been related to the developing purpura, which in turn was probably due, at least in part, to cytomegaly affecting the small blood vessels. (Thrombocytopenia was not present until the patient was moribund.) Quite apart from sensitization being considered as a symptom of Wegener's disease, sensitivity to drugs or to micro-organisms has been suggested as a factor in its causation. It is therefore pertinent to mention that some authors (Plummer *et al.*, 1957a) have suggested the need for special care in the use of chemotherapy and antibiotics in cases of this disease because they might prove more dangerous than useful.

There is no reason to believe that either cytomegalic inclusion-body disease or the concomitant pneumocystis infection caused or contributed to the development of Wegener's disease in this case: indeed, there are more cogent reasons for accepting the converse view, that the latter predisposed to the infections. Whether the antibiotics and prednisolone influenced the course of the infections is unanswerable. It is worth noting that steroids have been used as treatment for cytomegalic inclusion-body disease in infancy, being given because of their anticipated effect on the thrombocytopenia, haemolytic anaemia, and hepatitis which are associated with the severest form of the generalized congenital infection: success has been claimed for this treatment in three such cases (Margileth, 1955; Birdsong, Smith, Mitchell, and Corey, 1956; van Gelderen, 1959). While the cause of the anaemia and thrombocytopenia in the case reported here was obscure there may well have been, as in the severe congenital infections, more than just a chance association with the inclusion-body cytomegaly.

Case 2: Generalized Cytomegalic Inclusion-body Disease, Pneumocystis Pneumonia, Recurrent Cryptococcal Pneumonia, and Thrombotic Purpura

A 47-year-old man was admitted to a nursing-home because of acute haemolytic anaemia. His health had previously been good, apart from a lobectomy six years earlier following the chance radiological finding of a peripheral lung shadow: histological study had shown this to be a localized cryptococcal granuloma, and no sign of infection elsewhere was found. Eight weeks before his admission to the home with haemolytic anaemia he had an attack of acute pharyngitis (temperature 104° F. [40° C.]) during an outbreak of streptococcal infection. He was given oxytetracycline (total dose 5 g. in five days): the infection quickly subsided, but he was left feeling unexpectedly tired and weak. Examination of the blood on admission showed 2,000,000 erythrocytes per c.mm.; haemoglobin 6.0 g. per 100 ml.; leucocytes 6,900 per c.mm. (normal differential count); spherocytes and normocytes were conspicuous in the films. Coombs tests (direct and indirect) were negative. A series of blood transfusions had no significant effect on the haematological findings. Eight days after admission purpura began to appear; platelet count 34,000 per c.mm. He became drowsy and confused, with muscle twitching and transient episodes of choreiform movements. Blood urea 36 mg. per 100 ml.; serum bilirubin 4.8 mg. per 100 ml. Urine analysis showed protein +; urobilinogen +++; many erythrocytes in deposit. His temperature since admission had ranged between 99 and 101.8° F. (37.2–38.8° C.), and oxytetracycline was given throughout the first 14 days in the home (0.25 g. by mouth six-hourly, total dose 14 g.). On the eighth day it was realized that he had thrombotic purpura: steroid therapy was started (hydrocortisone 50 mg. by mouth thrice daily, total dose 2,100 mg. in 14 days), and it was decided to perform splenectomy without delay. At laparotomy no trace of the spleen was found; there were no splenunculi or haemolymph nodes, and no serosal or retroperitoneal splenosis. The opportunity to take tissue for histological confirmation of the diagnosis was missed. With the administration of hydrocortisone his condition began to improve. His temperature became normal, purpura faded, and the neurological and mental disturbances disappeared. Six blood transfusions, each of half a litre, were given during the eight days following the laparotomy: the erythrocyte count rose from 2,000,000 per c.mm. to 3,900,000 a fortnight later, and evidence of haemolysis disappeared. The platelet count also rose steadily, but more slowly, and did not pass 120,000 per c.mm. On the fourteenth day after the operation (11 weeks after the initial pharyngitis) he died suddenly.

Necropsy Findings.—The necropsy was limited by request to examination of the thoracic and abdominal viscera, with removal of no more material for histological examination than was essential for diagnosis. A coiled embolic thrombus, clearly a cast of the main veins of one leg, was impacted at the bifurcation of the main pulmonary artery. The lungs were hyperaemic, and there was a sparse, patchy consolidation in both; the consolidated foci were greyish red, dry, and mostly about 1 cm. in diameter, and they had no predilection for any particular part of the lungs.

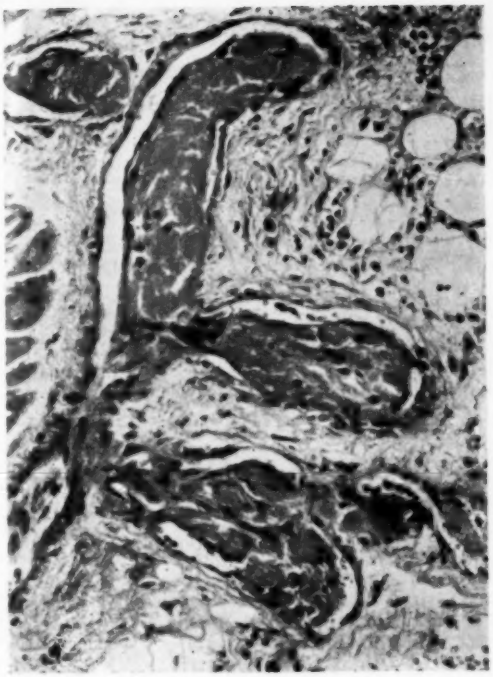

FIG. 23

Histological Findings. — Thrombotic microangiopathy (Fig. 23), typical of thrombotic purpura, was found in all the organs sectioned (heart, lungs, adrenal, and kidney). Inclusion-body cytomegaly was widespread in the lungs and adrenal, with bronchial ulceration and extensive adrenal cortical necrosis, as in Case 1; occasional affected cells were found in renal glomeruli; the section of heart (wall of left ventricle) was free from involvement. The consolidated foci in the lungs were typical of pneumocystis infection, with plasma-cell interstitial pneumonia and alveolar pneumocystosis (Figs. 21 and 22); pneumocystis was also occasionally found in the interstitial tissue itself, as described by Hamperl (1957). A single small focus of active torulosis was found in one of the four blocks of lung tissue which were sectioned (Fig. 24); it is not known from what part of the lungs this block came. No other lesions containing cryptococci were found.

It was impossible to obtain sections of the pulmonary lobectomy specimen for review.

Commentary on Case 2

All the criteria for the clinical diagnosis of thrombotic purpura were present in this case, and

FIG. 23.—Case 2: Myocardium. Typical thrombotic microangiopathy of thrombotic purpura. Haematoxylin-eosin. ×220.

FIG. 24.—Case 2: Lung. Cryptococcal pneumonia. Periodic-acid-Schiff; Mayer's haemalum. ×200.

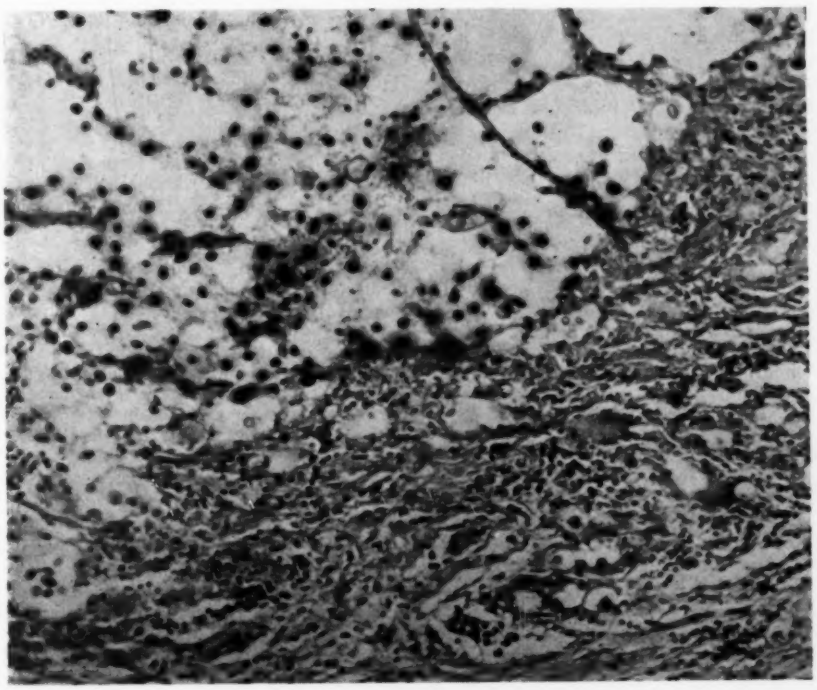

FIG. 24

this was confirmed by the typical histological findings *post mortem*. Although the number of acceptable published cases of thrombotic purpura is now over 150, the clinicopathological picture defined in the earlier studies has not been much amplified. The diagnosis is now made during life in a higher proportion of cases, and, although treatment is unavailing in the great majority of cases, large doses of steroids (with or without splenectomy) have been followed by long-lasting remissions in a few cases (Burke and Hartmann, 1959), and there is therefore some hope that the hitherto uniformly mortal outcome may at least occasionally be avoided. This might have been so in the case described here, in view of the remarkable remission, which, however, was cut short by the pulmonary embolism.

It is likely that the steroid therapy, and perhaps the antibiotics as well, acted in association with thrombotic purpura in this case, as with Wegener's disease in Case 1, to predispose to the generalized cytomegalic inclusion-body disease. The same factors may have been responsible for the reactivation of the cryptococcal infection, which, in view of the rarity of torulosis, is likelier to have been dormant in the lung since the lobectomy six years earlier than to have been a fresh infection.

The association of torulosis with pneumocystis infection has been recorded only once before (Winslow and Hathaway, 1959). The case was one of chronic lymphatic leukaemia, and the patient had been treated with *x* rays, prednisone, chemotherapy (nature not specified), and the instillation of radioactive gold into the body cavities. Necropsy showed leukaemia, disseminated torulosis, and pulmonary pneumocystosis. (I am indebted to Lieutenant-Colonel Winslow for the information that a histoplasmic granuloma was also found in this case when further sections of the lungs were examined.)

Thrombotic purpura, like Wegener's disease, has features which relate it to the group of collagen diseases, and this adds to the interest of the apparent role of these two diseases in predisposing to the infections described.

Agenesis of the spleen is very rare (Putschar and Manion, 1956); it is not likely to have been of aetiological significance in the development of thrombotic purpura, or of any of the associated infections. It is worth mentioning that in another case of thrombotic purpura (Symmers, 1956, Case 1) the spleen had been removed surgically because of traumatic rupture some years before the onset of this disease.

Case 3: Generalized Cytomegalic Inclusion-body Disease, Pneumocystis Pneumonia, Tuberculosis, and Hodgkin's Disease

The patient, aged 37, had had Hodgkin's disease for 18 years. This presented with unilateral cervical lymph-node enlargement, first noticed during medical examination for military service, and treated then by block dissection and deep *x*-ray therapy. The histological findings at that time could not be reviewed, but sections were available from a biopsy performed when cervical lymphadenopathy recurred six years later: this biopsy was originally described as "typical of Hodgkin's disease," but the picture was that which now is often known as the indolent (or so-called "benign") form of Hodgkin's disease ("Hodgkin's paragranuloma"). Deep *x*-ray therapy was followed by regression. A further six years later there was fresh lymph-node enlargement, this time involving cervical, axillary, and mediastinal nodes. A cervical node showed the typical histological picture of the ordinary form of Hodgkin's disease. Over the next five years the disease was with some difficulty kept under control by treatment with deep *x* rays interspersed with courses of cytotoxic drugs. The drugs used were mustine (nitrogen mustard), tretamine (triethylene melamine), chlorambucil ("leukeran"), and the folic-acid antagonist teropterin: each in turn produced a striking but short-lasting remission, each recurrence proving to be resistant to further administration of the drug last used. During the last year of his illness the patient's general condition became progressively poorer, and although he tolerated treatment less well courses of deep *x*-ray treatment and mustine were continued. Leucoerythroblastic anaemia developed, and repeated blood transfusions were needed. Three months before his death symptoms of subacute meningitis appeared. It was thought that this might prove to be due to torulosis, in view of the recognized liability of Hodgkin's disease to be complicated by this infection; however, repeated direct and cultural examinations of the cerebrospinal fluid showed tubercle bacilli and no cryptococci. No sign of tuberculosis was found clinically elsewhere; there was nothing to suggest tuberculosis in the chest radiographs, and no tubercle bacilli were found in the sputum. The meningitic symptoms subsided on treatment with streptoduocin (0.5 g. intramuscularly twice daily for five weeks, and then daily for three weeks, total dose 44 g.), isoniazid (25 mg. intrathecally daily for seven days, total dose 175 mg.), and sodium aminosalicylate (3 g. by mouth four-hourly for eight weeks, total dose 1,000 g.). His general condition continued to deteriorate in spite of the response of the meningeal infection; he died in a state of cachexia.

Necropsy Findings. — There was widespread involvement of the lymph nodes, spleen, liver, and bone marrow by Hodgkin's disease, with a few deposits in the kidneys, testes, and lungs. A small focus of partly excavated but fibrotic tuberculosis was

FIG. 25.—Case 3: Lung. Inclusion-body cytomegaly affecting several alveolar cells: typical "owl's eyes" appearance. Haematoxylin-eosin. ×1,000.

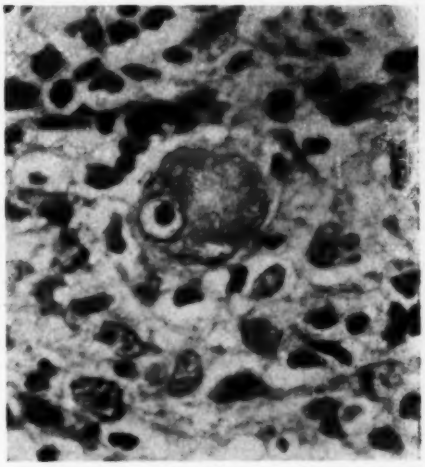

FIG. 26.—"Owl's eye" nucleus in a multinucleated giant cell in a lesion of Hodgkin's disease: such nuclei may be met with in Hodgkin's lesions in the absence of salivary-virus inclusion-body cytomegaly. Haematoxylin-eosin. ×600.

present in the apical part of one lung; an attempt to isolate tubercle bacilli from this lesion was not successful. There was widespread suppurative bronchopneumonia. No other macroscopical abnormalities were recorded. Permission to examine the brain and spinal cord was not obtained.

Histological Findings.—The biopsy findings have been summarized in the case history. The sections of the post-mortem material confirmed the naked-eye findings; the lesions of Hodgkin's disease were largely fibrotic, but the fibrous tissue enclosed many islands of typical, active Hodgkin's disease. Alveolar pneumocystosis was found in two of the six pieces of lung examined: plasma-cell and lymphocytic infiltrate was present in the septa in these foci, but was less abundant than in Cases 1 and 2. Inclusion-body cytomegaly was widespread, with particularly heavy involvement of the lungs (Fig. 25) and adrenals, as in the other two cases; bronchial ulceration and necrosis of the affected areas of adrenal cortex were particularly marked. Endothelial and interstitial cells in the spleen, lymph nodes, and bone marrow were commonly affected by this infection: in spite of the superficial resemblance between the nuclei of the atypical reticulum cells of the Hodgkin lesions and those affected by inclusion-body disease, there was no real difficulty in distinguishing the two.

Commentary on Case 3

Little need be said about this case, as most of the points which it illustrates have been covered in the commentaries on the preceding cases. The main difference from the other cases was that the patient was not given steroid therapy. On the other hand, in addition to streptomycin and repeated blood transfusions he had been heavily treated with cytotoxic drugs, which are known to lessen resistance to some forms of infection, and he had also had sodium aminosalicylate and isoniazid shortly before his death. Hodgkin's disease, in common with other malignant diseases arising in the lymphoreticular system, has been recorded before in cases of cytomegalic inclusion-body disease, of which it is presumably one of the predisposing causes, as it is of torulosis (see Symmers, 1957). The finding of tuberculosis and not torulosis as the cause of the meningitis should not have been unexpected, for the liability to tuberculosis of patients with Hodgkin's disease was well known long before the comparable liability to torulosis and other fungal infections was recognized.

Discussion

The importance of these three cases of cytomegalic inclusion-body disease is not the rarity of this infection in adults but its severity and the circumstances of its occurrence. Although its effect on the course of the underlying disease cannot be defined, it should be looked upon as a grave complication. This is suggested, for instance, by the extent of the adrenal changes (which in Cases 1 and 2 were possibly complicated

by adrenocortical necrobiosis associated with steroid therapy), and in Case 1 by the severe melaena from the colonic ulcers and by the purpura. The extent to which the underlying disease and the treatment with antibiotics, steroids, or chemotherapy predisposed to the infection cannot be assessed; it is likely that the effect of each was enhanced by the other, although it is not known by what mechanism resistance can be so altered that an infection which seems ordinarily to occur only in babies, and then usually as a trivial infection confined to salivary glands, can become established as a dangerous generalized disease in adults. The diseases which have been recorded as predisposing to cytomegalic inclusion-body disease and its concomitant pneumocystis pneumonia include leukaemias and refractory anaemias, as well as Hodgkin's disease (and some other diseases of the lymphoreticular system): it is not surprising to find that diseases like Wegener's granulomatosis and thrombotic purpura, belonging to the group of disorders of the connective tissues, may also be liable to these infective complications. The diseases which predispose to these two infections are known also to predispose to some other unusual infections (torulosis, mucormycosis, toxoplasmosis, *Pasteurella septica* infection), and also to unusual disseminated forms of infection (tuberculosis, leprosy, moniliasis, aspergillosis, histoplasmosis) which in their usual manifestations are more or less commonplace in the communities to which the patients belong. It is relevant that certain therapeutic agents, including antibiotics, steroids, and some other drugs, e.g., sex hormones and cytotoxic drugs, and also radiotherapy, may likewise predispose to these unusual or atypical forms of infection. There is also experimental evidence, for instance, that steroid administration predisposes to pneumocystis pneumonia (Weller, 1955; Ricken, 1958).

As well as considering the possible interaction of two predisposing factors (the underlying disease and the treatment given) it is necessary to consider a third fundamental aetiological factor, the portal of entry of the infection. It is possible that the salivary virus is already present in dormant state in the tissues: it is likely that in man, as in animals, part of the population carries the virus, and that most of its hosts have enough immunity not to become ill. By analogy with other infections, an alteration in the host's immunological state might permit the virus to multiply, and the characteristic cell changes would then appear.

It is commonly stated that the salivary glands are not affected in adults with inclusion-body cytomegaly, in contrast with the sialotropism of the infection in babies. In most of the published cases in adults there is no mention of histological study of salivary glands, and in the cases reported in this paper no salivary glands were sectioned. Regular sectioning of tissue from the submandibular gland or, preferably, the parotid, in cases of this infection in adults is needed before a factual assessment of their supposed freedom from involvement can be made.

It has been suggested that the virus of inclusion-body cytomegaly may have been transmitted by blood transfusion in some of the published cases of this disease in adults, particularly as seven of these patients had blood diseases necessitating repeated transfusions. The histological findings in the mastoidectomy specimen in Case 1 showed that the infection was already present before the patient had any transfusions. For various reasons this route of infection, while possible, seems to be an unlikely one. If the infection is exogenous, and not the result of reactivation of a dormant virus in the patient's tissues, the likeliest route would presumably be droplet infection of the respiratory tract.

In all three of the cases reported here there was considerable ulceration of the bronchi, and in Case 1 there were also colonic ulcers; all of these ulcers were heavily affected by inclusion-body cytomegaly. There was no evidence that the ulceration was due to any of the associated diseases, and it is possible, although unproved, that they resulted directly from the viral infection. If this were the case they represent a possible portal of entry for the infection.

With regard to the recognition of cytomegalic inclusion-body disease, the only diagnostic difficulty arises in interpreting the nature of the alteration in those cells in which intracytoplasmic inclusions do not form, particularly histiocytes or other interstitial cells in the connective tissues. Occasionally, as in the intestinal muscle coats in the vicinity of the colonic ulcers in Case 1, the histological appearances may somewhat resemble carcinomatous infiltration until studied under high magnifications. It is also worth a mention that the cell affected by inclusion-body cytomegaly has a superficial likeness to the atypical reticulum cells of reticulosarcomas and of Hodgkin's disease, with their large nucleus, distinct nuclear membrane, clear nucleoplasm, and unusually conspicuous nucleolus (Figs. 25 and 26).

The failure to recognize the cytomegaly in the mastoid tissue in Case 1 until the biopsy sections were reviewed, after the diagnosis had been made *post mortem*, indicates the need for the histologist to be on the look-out for this disease in biopsy material. It will be interesting to learn whether generalized cytomegalic inclusion-body disease is met with oftener as the present emphasis on treatment with antibiotics, chemotherapeutic drugs, and steroids continues. The infection is likelier to have its main practical importance in cases of diseases like sarcoidosis than in those which in any case are likely to prove mortal. In sarcoidosis, when appropriately treated, the prospect of recovery should be good: but sarcoidosis, like Hodgkin's disease, affects the lymphoreticular system predominantly, and therefore can seriously interfere with the body's defences against infection. By analogy with Hodgkin's disease, and with its own liability to predispose to torulosis and some of the other infections noted above (Plummer *et al.*, 1957b), sarcoidosis may prove to be one of the diseases which predispose to generalized cytomegalic inclusion-body disease, particularly if the defences have been further weakened by the effects of drugs.

It is a pleasure to express my thanks to my colleagues, Dr. H. S. Baar, Dr. M. Bodian, Dr. W. E. D. Evans, Dr. N. E. France, Professor H. Hamperl, Dr. N. S. Plummer, Dr. G. A. C. Summers, and Mr. Lionel Taylor, for allowing me access to their records and material in the preparation of this paper, for comparative material from other cases, and for permission to publish the case histories and necropsy reports. My thanks are also due to Mr. F. D. Humberstone, Mr. K. R. James, and Mr. K. W. Iles for their technical and photographic collaboration.

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CLINICAL AND PATHOLOGICAL FINDINGS IN OCHRONOSIS

BY

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A case of ochronosis with far-advanced cardiovascular, skeletal, and prostatic manifestations is reported. An attempt is made to correlate the location of pigment with other pathological changes in this disorder. A brief discussion of the hereditary factors, the metabolic pathways of homogentisic acid, and the pathogenesis of ochronosis is presented.

Ochronosis frequently presents as a triad of arthritis, dark urine, and bluish discoloration of cartilage. Angina is a late manifestation, and necropsies have demonstrated considerable atherosclerotic changes in the intima and media of the coronary arteries (Lichtenstein and Kaplan, 1954). The following case seems worthy of presentation because of the unusual aspects of coronary thrombosis and prostatic calcification, which clinically resembled carcinoma of the prostate. In addition the pathological distribution of pigment suggested certain theoretical considerations concerning the deposition of homogentisic acid which will be discussed.

Case Report (No. 15008)

A 54-year-old white man was admitted to the hospital on August 4, 1956, because of increasing exertional chest pain and dyspnoea.

He had noted that his underclothing had been stained with black deposits from his urine since childhood, and, in 1937, a peculiar discoloration of the ears and sclera was observed. At the age of 29 he spent a short time in hospital because of a back injury. He was thereafter asymptomatic until 1941 when he developed persistent low back pain radiating into the right leg which was aggravated by jarring movements. In 1945 he was examined for arthritis of the back and legs, diagnosed as having ochronosis, and was medically retired from the armed forces. Because of ankylosis of the right knee, he was in hospital in 1950, at which time his right leg was treated with casting and arthroplasty. A three months' course of A.C.T.H. gave partial relief.

In November, 1954, he was first admitted to the Veterans Administration Hospital because of pain and stiffness in the neck, shoulders, and arms, and an exacerbation of the right leg and back pain. The blood pressure was 164/110 mm. Hg. The diagnosis of alkaptonuria with ochronosis was confirmed. Because the prostate was enlarged, hard, asymmetrical, painless, and fixed, a needle biopsy was necessary and revealed ochronotic calculi with no evidence of tumour. Subsequently during cystoscopy a large, irregular, black, staghorn calculus was seen in the prostatic urethra and removed. Furthermore, transurethrally resected prostatic specimens contained numerous, small, black calculi within acini which were consistent with ochronotic involvement of the prostate with no evidence of carcinoma. In March, 1955, a calculus was removed from the tip of the urethra by meatotomy.

In June, 1955, he first developed exertional dyspnoea and chest pain which radiated into the shoulders and arms. He also noted nocturnal dyspnoea. On September 21, 1955, he was awakened by a severe, crushing, precordial chest pain radiating into both shoulders and down the ulnar aspects of the arms, associated with dyspnoea and diaphoresis. This pattern recurred the following day, requiring the patient's admission to the hospital, where electrocardiographic evidence of acute myocardial infarction was obtained. The serum glutamic oxalacetic transaminase level was 112 units. The course was uneventful except for thrombophlebitis of the left leg, and he was discharged on December 22, 1955, on digitalis, dietary sodium restrictions, vasodilator drugs, and 25 mg. of bis-hydroxycoumarin per day. Subsequently he had angina of effort three to four times a day, attacks which increased during the following winter months. On April 17, 1956, he was again admitted to hospital because of increasing chest pain, orthopnoea, and a moderate cough.

Physical Examination.—The patient was a short, fairly well-developed, obese, white male who appeared chronically ill. The aural pinnae and ridge of the nose were bluish-grey. The lateral portions of the sclerae contained dark brown pigmentation. Ophthalmoscopic examination revealed moderate AV-nicking and arteriolar attenuation and tortuosity.

The neck was hyperextended and there was marked limitation of all cervical movement. The thoracic cage was fixed in inspiration with marked dorsal kyphosis, slight dextroscoliosis, and increased anteroposterior diameter. The blood pressure was 130/90 mm. Hg with a regular heart rate of 92 per minute. The point of maximum impulse was displaced to the left and there was a Grade II, blowing, systolic, apical murmur transmitted into the aortic area. The prostate was hard, fixed, asymmetrical, and not tender. Popliteal and dorsalis pedis arterial pulsations were palpable. The lower extremities were atrophic, markedly deformed, and limited in movements.

Laboratory Findings.—In the course of his several admissions, the total blood count, erythrocyte sedimentation rate, Kolmer, serum albumin and globulin, serum cholesterol and esters, glucose tolerance, cephalin cholesterol flocculation, alkaline and acid phosphatase, inorganic phosphorus, blood urea nitrogen, and serum uric acid had been within normal limits. Urine analyses were acid in reaction, with specific gravities up to 1.023 with normal urinary sediments. On standing, the urine became progressively brown and turned black on alkalinization. Quantitative analysis revealed 4.3 g. of homogentisic acid per litre of urine. Cerebrospinal fluid total protein was 59 mg.%.

FIG. 1.—Lateral view of the lumbo-sacral spine showing narrowed joint spaces, osteoporotic vertebral bodies, and marked calcification of the intervertebral discs and ligaments.

Radiographs revealed marked degenerative changes in the knees, sacro-iliac, hip, and shoulder joints. Films of the thoraco-lumbar spine demonstrated osteoporotic vertebral bodies and marked calcification of the intervertebral ligaments and of the intervertebral discs, findings which were thought to be diagnostic of ochronotic arthritis (Fig. 1).

An electrocardiogram showed changes of an acute anterior inferior myocardial infarction on September 26, 1955, with evolutionary changes noted on subsequent tracings.

During the final admission the patient was placed at bed rest on a sodium-restricted diet and given digitalis, vasodilators, and anticoagulant therapy. Transitory improvement was followed on April 19, 1956, by cardiac asystole and sudden death despite emergency treatment.

Necropsy Findings.—External examination of the body revealed arthritic deformity of all the joints. The skin was normal except over cartilaginous areas, where blue-black discoloration was visible. Dissection of the musculature from the chest wall revealed blackish discoloration of the costal cartilages (Fig. 2).

The heart (weight, 550 g.) revealed calcific atheromatous plaques in the aortic and mitral valve cusps and similar plaques throughout the aorta and coronary arteries. These plaques were blue-black and similar pigmentary changes occurred in the chordae tendineae. There was old and recent infarction of the anterior wall of the left ventricle, the anterior portion of the interventricular septum, and the apex of the heart secondary to thrombosis of the anterior-descending branch of the left coronary artery (Fig. 3).

The lungs were oedematous and congested. The tracheal and bronchial cartilages were discoloured, mainly at the periphery.

The surfaces of the kidneys were pitted and irregular. On section, the medulla was reddish-black and the arcuate arteries were outlined by black pigment. The urine in the bladder was dark and the mucosa was bluish-black. Black calculi were present in grossly dilated acini of a hard and fibrous prostate.

The bony trabeculae of the sternal marrow, the intervertebral disc cartilages, the cartilage and synoviae of the knee joint, and the quadratus femoris tendon were black and brittle (Fig. 4).

The vessels in the dura and the brain were outlined by black pigment deposits.

Microscopy of the heart revealed an acute infarct superimposed on areas of fibrosis. Sections of the coronary arteries and aorta revealed brown, granular pigment in the endothelial cells and also in calcified atheromatous plaques (Fig. 5). Similar endothelial and perivascular pigmentation was seen in the lungs and other viscera with endothelial pigmentation, especially marked in the spleen, adrenals, the arcuate arteries of the kidneys, and submucosal vessels of the

FIG. 2.—Under-surface of the sternum. The costochondral cartilages are markedly pigmented.

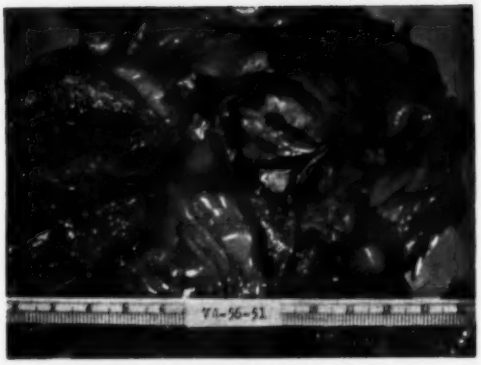

FIG. 3.—Pigmented areas are seen in the thrombus of the coronary vessels and in the region of the aortic valves.

FIG. 4.—Sagittal section of the lumbosacral vertebra. On the right is a view of the articular surface of the right knee joint, which shows similar pigmentation.

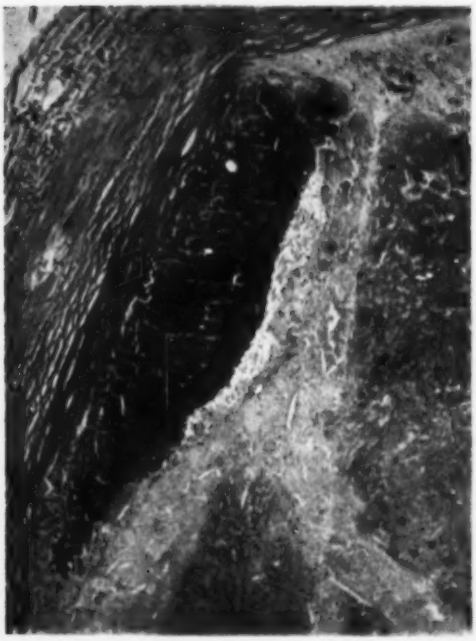

FIG. 5.—Section of the occluded artery ×150. A pigmented atheromatous plaque is located at the site of the occluding thrombus.

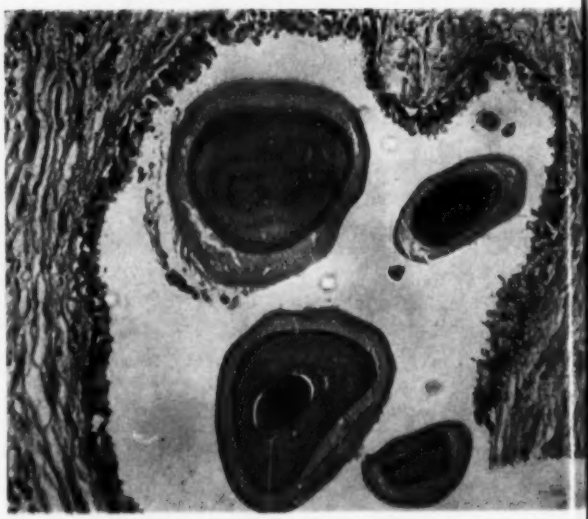

FIG. 6.—Section of prostatic acinus ×150. The dilated acinus contains pi[illegible] calculi and corpora amylacea.

urinary bladder. In the perivascular tissue, granular pigment was prominent and occurred both within histiocytes and free in the extracellular spaces. Liver parenchymal cells were pigmented, as was renal tubular epithelium.

The prostate was fibrous and pigmented calculi and corpora amylacea were numerous within dilated acini (Fig. 6).

The villous synovium of the knee showed fibrosis and calcification occurring about extracellular brown pigment granules. Here again, endothelial cells were heavily pigmented. In skeletal structures, the heaviest pigmentation occurred beneath periosteum, perichondrium, and in those vascular areas subject to trauma. Cartilage and bone architecture, however, remained essentially normal despite pigmentation.

Discussion

Pathologically it appears that there are two primary sites of pigment deposition in this patient with ochronosis, namely vascular endothelium and the organs of excretion. Liberation of granules from the endothelial cells may be capable of causing the degenerative changes seen in this disorder of metabolism. These include the hypertrophic arthritis and atherosclerosis. Excretion of homogentisic acid accounts for pigmentation in many other sites such as liver, sweat glands, renal tubules, prostatic acini, and bladder mucosa.

The pathological findings in this patient with ochronosis correlate with those previously documented in the medical literature (Lichtenstein and Kaplan, 1954; La Du, Seegmiller, Laster, and Zannoni, 1958a and b; Haldane, 1941; Galdston, Steele, and Dobriner, 1952; Garrod, 1908, 1923; Oppenheimer and Kline, 1922). However, the usual changes in the skin due to pigment deposits in endothelial cells of the dermal vessels and epithelial cells of sweat glands (Lichtenstein and Kaplan, 1954) were not found in our patient.

The patient showed the cardiovascular pigmentation in the endothelial cells, intima, media, and perivascular tissues (Lichtenstein and Kaplan, 1954; Oppenheimer and Kline, 1922) as previously reported. These changes appear to accentuate atherosclerosis. In fact, roughened intimal plaques were the site of thrombus formation and were the cause of myocardial infarction.

Cartilage pigmentation and secondary hypertrophic arthritic changes were especially prominent in the knees, spine, and shoulders, and were similar to those reported elsewhere in the literature (Sutro and Anderson, 1947; Kolaczek, 1910; Pomeranz, Friedman, and Tunick, 1941). In cartilage the greatest deposits occur peripherally beneath the perichondrium. With pigmentation of the cartilage, it becomes brittle and breaks when submitted to stress, leading to traumatic arthritis due to free joint bodies. Synovial reaction occurs secondarily to trauma and some fibrosis also in the synovial tissues in response to extracellular granular pigment.

Pigmentation of arcuate arterial endothelium and also of renal tubular epithelium has been documented previously (Galdston *et al.*, 1952), and also occurs in this case. Prostatic calculi and corpora amylacea are pigmented and appear to result from excretion of homogentisic acid by this organ (Young, 1944). The calculi and secondary fibrosis were responsible for the clinical findings suggestive of carcinoma. The need for biopsy proof of carcinoma is further substantiated, and this particular case illustrates the limits of digital examination.

Pigment has been reported to occur in various endocrine structures of the body, especially the pancreatic islets, pituitary gland, and the pineal body (Lichtenstein and Kaplan, 1954; Galdston *et al.*, 1952; Garrod, 1908, 1923; Oppenheimer and Kline, 1922; Boyd, 1947).

Garnier and Voirin demonstrated that homogentisic acid, an intermediate metabolite of phenylalanine and tyrosine, is completely metabolized by normal individuals (Garnier and Voirin, 1892). Alkaptonurics were shown to have an inability to metabolize compounds at the 2,5 positions on the benzene ring of homogentisic acid. The metabolic defect of alkaptonuria (hereditary ochronosis) lies in this inability and is the result of a single recessive Mendelian factor (gene) mutation. This alters the ability of the cell to produce the enzyme (homogenticase, homogentisic oxidase) responsible for the degradatory oxidation of homogentisic acid to fumaryl acetoacetic acid (La Du *et al.*, 1958a and b). Other mutative changes are involved in the failure to convert phenylalanine to melanin (albinism); inability to transpose the hydroxyl groups of 3,4 dihydroxyphenylalanine to the 2,5 positions (tyrosinosis); and the loss of the enzyme necessary to oxidize the *p*-hydroxypyruvic acid to the 2,5 dihydroxy form (phenylpyruvic oligophrenia).

Garrod (1908, 1923) first recognized and described the hereditary nature of ochronosis in 1904, and noted the high frequency of consanguineous marriages in family pedigrees. He explained the pathological findings by the concept of "inborn errors of metabolism,"

postulating a genetically controlled absence of an enzyme. Others have corroborated Garrod's findings (Hogben, Worrall, and Zieve, 1932).

The pathological changes seen in ochronosis have been thought, by previous authors (Lichtenstein and Kaplan, 1954; La Du *et al.*, 1958a and b; Haldane, 1941; Galdston *et al.*, 1952; Garrod, 1908, 1923; Oppenheimer and Kline, 1922; Sutro and Anderson, 1947), to be the result of accumulation of homogentisic acid and its polymers in tissues such as cartilage, fibrocartilage, tendons, and the supportive fibrous tissues of the arteries and of the heart. The special affinity of these tissues was thought to be enhanced by the high concentration of homogentisic acid. Physical stress may conceivably cause injury to cells of vascular tissue with disruption of the cellular membrane and liberation of pigment granules, which may be capable of promoting degenerative changes in the adjacent perivascular spaces (Boyd, 1947). The presence of foreign material within a relatively anoxic tissue greatly enhances fibrosis. Contraction of the fibrous tissue may result in additional anoxia, tissue destruction, and progression of the pathological changes demonstrated in ochronosis. Such a theory of stress in an area of relatively poor vascularity can explain the changes which are so marked in the cartilages, tendons, and especially within the skeletal joint spaces.

Illustrations by Frank Roberts and John Friel, Medical Illustration Service, Veterans Administration Hospital, Omaha, Nebraska.

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THE PATHOLOGY OF ASTHMA, WITH SPECIAL REFERENCE TO CHANGES IN THE BRONCHIAL MUCOSA

BY

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The pathological features of 20 cases dying in status asthmaticus have been studied. In gross sections the lungs showed no emphysema, but mucus plugs in the air passages and focal areas of collapse were outstanding features. Five cases showed cystic bronchiectasis which was of a similar distribution to the focal areas of collapse, occurring in the upper lobes as commonly as in the lower lobes.

Histologically, shedding of the ciliated bronchial mucosal cells was prominent and this is attributed to a transudation of oedema fluid from the submucosa. Areas of regeneration of the mucosa, with the presence of simple stratified epithelium, were seen frequently. The loss of the ciliated respiratory epithelium and the transudation of oedema fluid into the bronchial lumen, with interference with the action of the remaining ciliated cells, are considered to be the essential factors in the failure of clearance of the bronchial secretions in asthma. It is postulated that bronchospasm plays little or no part in the shedding of the bronchial mucosa or in the pathogenesis of the asthmatic attack.

As long ago as 1883 Curschmann noted the presence of ciliated columnar respiratory cells in the sputum of patients suffering from asthma. Ellis (1908) observed detachment of the bronchial mucosa, with consequent ulceration of a blood vessel, in a necropsy on a patient dying in status asthmaticus. Houston, De Nevasquez, and Trounce (1953), in a clinico-pathological study of nine fatal cases of asthma, mentioned partial or complete detachment of the bronchial mucosa as a constant finding. The epithelium was intimately mixed with mucus and eosinophils and assisted in plugging the bronchial lumen. They considered that this detachment of the epithelium was due to a mechanical separation consequent upon contraction of the bronchial muscle. The crenation of the bronchial wall was taken as evidence of muscle spasm, but the illustrations purporting to show this were not altogether convincing. It was to see if some other more logical explanation of the necropsy findings in asthma could be found that the following series of cases was studied.

Material

Lungs collected at necropsy from 20 patients dying in status asthmaticus have been examined. The lungs were fixed by perfusion with 10% formol saline through the main bronchus or, where this was not feasible because the bronchus was completely blocked by mucus, through the pulmonary artery. Up to 20 blocks of tissue were examined from each lung, and sections were stained with haematoxylin and eosin, Weigert's haematoxylin and Van Gieson, by

TABLE I
CLINICAL DETAILS OF CASES

Case No.	Age (yr.)	Sex	Duration of Disease (yr.)	Family History	Special Features in Lungs
1	10 mths.	F	2 mths.	Nil	—
2	4	F	1½	+	—
3	10	F	6½	Nil	—
4	15	F	13½	+	—
5	21	F	19	+	—
6	24	F	21	+	—
7	25	F	22	Nil	—
8	27	M	25	,,	Bronchiectasis and scattered nodular fibrosis
9	28	M	26	+	—
10	29	M	25	+	—
11	34	M	9	+	Bronchiectasis
12	38	F	32	Nil	—
13	41	F	Many years	,,	—
14	43	F	4	,,	—
15	44	F	30	+	Bronchiectasis
16	50	M	18	+	—
17	54	F	27	Nil	Bronchiectasis
18	56	M	4	,,	—
19	64	F	3	,,	—
20	67	F	30	,,	—

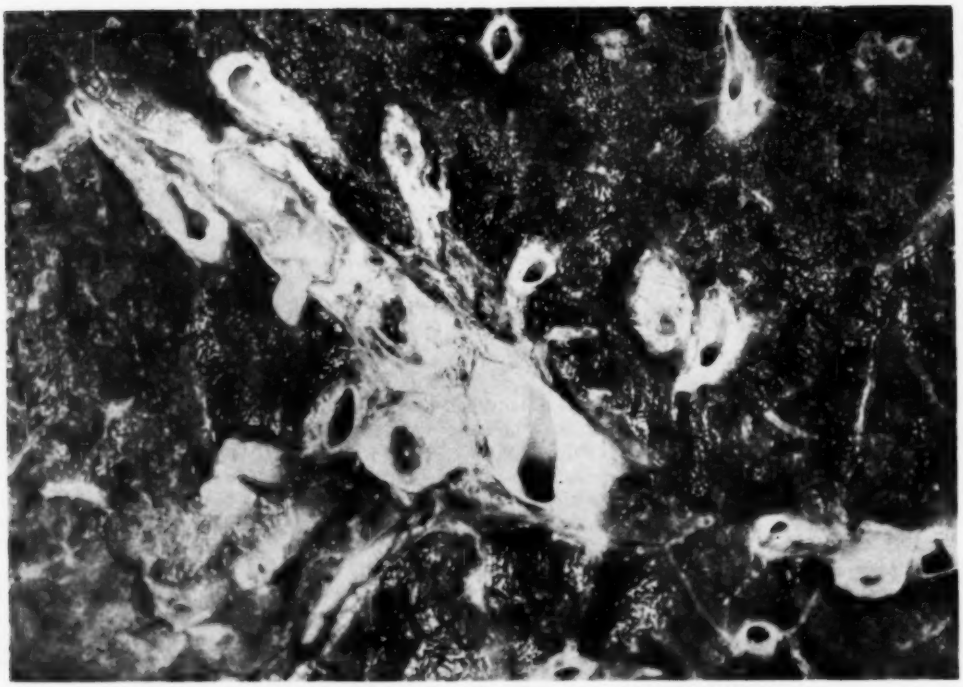

FIG. 1.—Cut surface of lung from Case 6 showing mucus plugs in medium-sized bronchi, × 1.

the periodic-acid-Schiff method, by Robb-Smith's reticulin method, with Masson's trichrome stain, and with phosphotungstic acid haematoxylin. Some clinical details of the cases are given in the table.

Gross Findings in the Lungs

The appearances of the lungs at necropsy when death has resulted from status asthmaticus are characteristic. On opening the thoracic cage it is at once apparent that both lungs are acutely distended. They fill the chest, completely covering the pericardium, nearly meeting in the midline anteriorly, and failing to collapse once the negative intrathoracic pressure has been released. Gough (1955) has likened them to the lungs in drowning. In the cases examined in this series, bullous, centrilobular, or focal emphysema was not found in a single instance. A frequent but not invariable finding was the presence of small focal areas of collapse, and these were seen in Cases 1, 2, 3, 5, 7, 10, 11, and 18. They were not confined to one surface or lobe of the lung but were seen just as frequently along the anterior margins and at the apices as at the bases. They appeared as firm, dark, airless, segmental areas depressed below the level of the distended lung.

The cut surface of the lung showed a striking picture with numerous grey, glistening mucous plugs scattered throughout the air passages (Fig. 1). These plugs were always seen in the small bronchi, but in some cases extended up to and occluded a main bronchus. In Case 8 both main bronchi were tightly packed with viscid mucoid material, and, in spite of the fact that intratracheal perfusion with formol saline under a pressure of 5 feet of water was attempted, little or none entered the lung. More often, however, a few segments became distended by fixative while the rest of the lung stayed distended with air.

In Cases 8, 11, 15, and 17 areas of cystic bronchiectasis were present. The asthmatic is peculiarly prone to develop bronchiectasis, because if a bronchus is completely occluded absorption collapse of the lung distal to it will result. Bronchiectasis quickly follows this state, as Lee Lander and Davidson (1938) have shown. Evidence that this is the mechanism involved in the production of bronchiectasis in asthmatics is

FIG. 2.—Cut surface of lung in Case 15 showing cystic bronchiectatic spaces in upper lobe and apex of lower lobe, × ½.

provided by the distribution of the lesions in the lungs studied. The upper and middle lobes or apex of the lower lobe were as often affected as the basal segments of the lower lobes (Fig. 2); the lesions thus followed a distribution similar to the focal areas of collapse already mentioned. The changes seen in the bronchi described in this paper do not, however, concern the mucosa of the bronchiectatic spaces. One further point of interest with reference to the cases showing bronchiectasis is that these were the only cases with any degree of right ventricular hypertrophy. Liebow, Hales, and Lindskog (1949) have shown that large precapillary bronchopulmonary arterial anastomoses occur in bronchiectasis, and it has been suggested that this is an important factor in the causation of pulmonary hypertension and right ventricular hypertrophy in this condition.

Histology

Lumen of the Respiratory Passages.—The most striking feature in any section from an asthmatic lung is the presence of a dense exudate in the bronchial lumen (Fig. 3). This can be seen as an occluding plug in the segmental bronchi, but it may extend distally as far as the respiratory bronchioles. The appearance of the exudate in haematoxylin-and-eosin preparations is somewhat variable. There is a mass of basophilic mucoid material, in which spirals of cells are entwined, but often areas of the exudate have a more eosinophilic appearance. Sections stained by the periodic-acid-Schiff method show that only the basophilic parts of the exudate are strongly positive. The eosinophilic part of the exudate is presumably proteinacious in character; it does not give the staining reactions of fibrin with phosphotungstic-acid-haematoxylin or Masson's trichrome.

The cellular content of the exudate consists of eosinophils which are scattered fairly evenly throughout the mucoid portion and some epithelial cells. The possibility of the epithelial cells being a post-mortem artefact was considered, as these cells undoubtedly do become detached easily after death, particularly if fixation is inadequate. The idea was rejected, however, as in places the cells are so intimately mixed with mucus. In addition they sometimes appear in the centre of the exudate in a bronchus with a normal lining mucosa indicating that they have come from another part of the bronchial tree. These mucosal cells appear singly or in groups, and they may be normal ciliated columnar cells or goblet cells.

Another point in connexion with the exudate worthy of note is the paucity of neutrophil polymorphonuclear leucocytes. Histologically in none of the cases, including those with bronchiectasis, was there evidence of acute pyogenic infection.

Mucous Membrane of the Bronchi.—In some areas the bronchial mucosa may appear normal, but in those bronchi plugged with exudate there are obvious pathological changes. Commonly

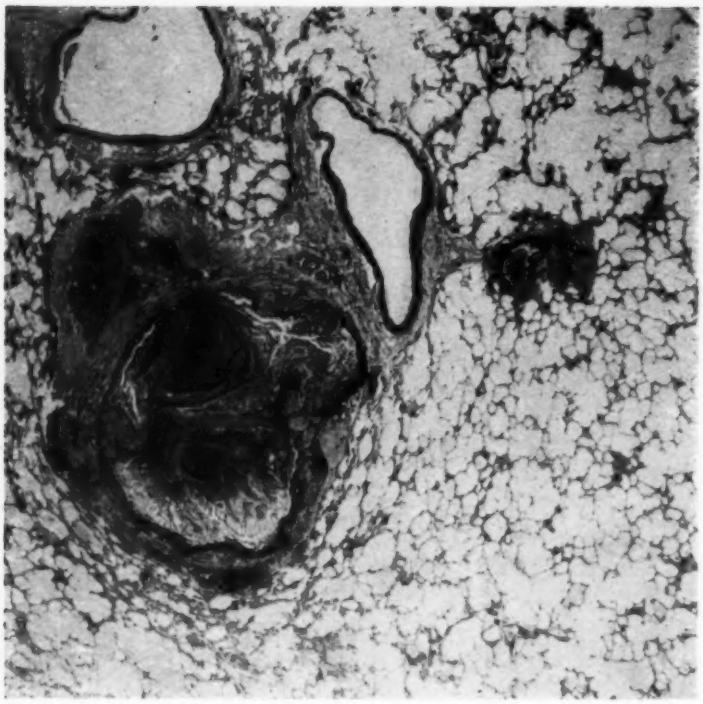

FIG. 3.—Low-power view showing bronchus plugged by exudate in Case 7. Note the absence of emphysema in the surrounding lung. Haematoxylin and eosin, × 14.

there is a marked degree of mucosal oedema with separation of the superficial columnar cells (Fig. 4). Eventually these cells become detached, and the remaining mucosa consists only of a few reserve or basal cells (Fig. 5). More interesting still are areas of regeneration which can be seen at focal points in the bronchial wall. Mitotic activity among the deeper cells is increased, and the epithelium is of the simple stratified type (Figs. 6 and 7). These areas were seen in all cases, but were much more marked in some than others. In Case 8 all the bronchi examined were lined by this metaplastic or regenerating epithelium. The appearances are very reminiscent of those seen by Wilhelm (1953) and Otto and Wagner (1956) when the trachea and bronchi were undergoing repair following experimental trauma. In the asthmatic lung these areas represent places where the mucosa has become detached and repair is taking place. All stages can be seen, from areas where only a few basal cells are present, through areas of metaplasia with simple stratified epithelium, to the normal pseudo stratified columnar, ciliated, respiratory epithelium. These metaplastic cells have recently been detected in the sputum of asthmatics by Cohen and Prentice (1959).

Eosinophils can be seen occasionally in the intact but oedematous bronchial mucosa, and presumably diapedesis of these cells occurs through the mucosa.

One of the most pronounced changes in the bronchial wall is the thickening of the basement membrane. This was seen in all except the two youngest patients in the series, Cases 1 and 2. It presents as a thick eosinophilic band to which the cells of the bronchial mucosa are closely applied. The membrane stains red in van Gieson preparations, green with Masson's trichrome, and pink with the P.A.S. technique. In sections stained for reticulin it is black, but sometimes, particularly in those cases where it is greatly thickened, it has a brownish collagenous component. In chronic bronchitis the basement membrane becomes thickened, but thickening is more variable and never so pronounced as in asthma.

Beneath the basement membrane, apart from the infiltration by eosinophils, the most striking feature is the dilatation of the capillary blood

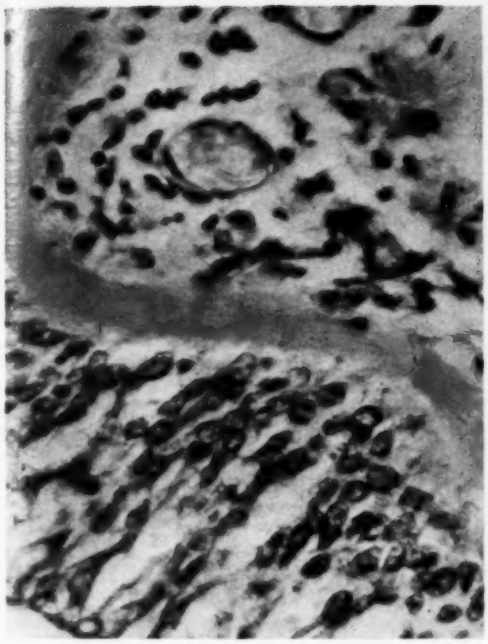

FIG. 4.—Section of a medium-sized bronchus in Case 7, showing mucosal and submucosal oedema. Haematoxylin and eosin, × 400.

FIG. 5.—Section showing a medium-sized bronchus lined by reserve or basal cells only. Columnar and ciliated cells are present in the exudate. Haematoxylin and eosin, × 112.

IG. 6.—Section showing medium-sized bronchus lined by simple stratified epithelium. Haematoxylin and eosin, × 400.

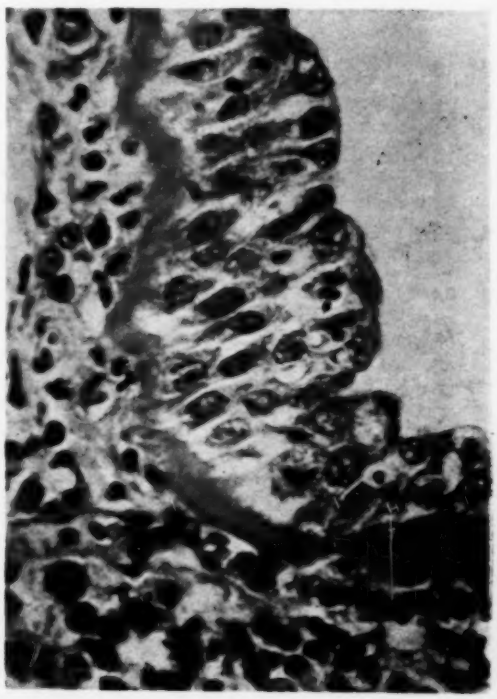

FIG. 7.—Commencing redifferentiation of bronchial epithelium with marked mitotic activity. Note the thickened basement membrane. Masson's trichrome, × 400.

vessels. These vessels, often possessing rather swollen endothelial cells, are closely applied to the basement membrane. The connective tissue in which these vessels lie consists of strands of widely separated collagen giving the impression of oedema (Fig. 4). Furthermore, the mucosa overlying these areas often shows oedema with separation of the cells. It is interesting to note that Wilhelm (1953) found that prominent submucosal inflammation, following removal of the tracheal epithelium, prolonged mitotic activity in the regenerating epithelium and delayed its differentiation.

The Bronchial Wall.—The mucous glands appear active but not hypertrophied as they are in chronic bronchitis (Reid, 1954).

The smooth muscle in the walls of the bronchi, especially those of 0.4–0.6 mm. diameter, shows considerable hypertrophy. Crenation of the bronchial mucosa of the type seen in animals dying of anaphylactic shock (Dixon and Warren, 1950), and said to indicate bronchospasm, was not noted. Indeed, the smaller bronchi and the bronchioles appeared if anything rather dilated (Fig. 3) with the bronchial wall stretched over the dense exudate in the lumen. In some of the large air passages it is true that irregularities can be seen in the bronchial mucosa with the formation of diverticula. Frequently these represent the mouths of the mucous gland ducts, and this can be demonstrated by serial sections. In other cases they may possibly represent the contraction of the bronchial smooth muscle, but even so it is difficult to credit that they would affect the diameter of the air passages in which they are present.

Discussion

In a recent lecture Young (1959) cited three main factors in the production of an asthmatic attack: (1) Spasm of the smooth muscle of the bronchi, (2) swelling of the mucous membrane, and (3) production of excessive glairy mucus. Clinically the concept of bronchospasm depends upon the auscultatory sign of a high-pitched expiratory rhonchus or wheeze. This is a sound produced in an air passage due to partial occlusion of its lumen, and there is nothing in the sound itself to differentiate obstruction by oedema of the bronchial mucous membrane, or by mucus, from spasm of the bronchial muscle. Anatomical evidence that asthma is due to bronchospasm is based, first on the presence of hypertrophied smooth muscle in the bronchial wall, and secondly on the doubtful finding of the crenation of the bronchial mucosa.

There is ample evidence for the allergic origin of bronchial asthma in its association with hay fever and urticaria, the frequency of eosinophilia in the peripheral blood, and positive skin sensitivity tests to foreign proteins. Segal and Attinger (1957) have stated that the allergic reaction is associated with (1) capillary dilatation, (2) increased capillary permeability, (3) increase in the protein content of intercellular fluid, (4) engorgement of lymphatic and blood capillaries, (5) accumulation of eosinophils, and (6) accumulation of mucus secretion in the walls of the bronchioles. The anatomical counterparts of these six points can be clearly seen in the submucosa of the bronchi where there is oedema and capillary dilatation.

Pathologically the outstanding feature of the asthmatic lung lies in the failure of clearance of the bronchial secretions. Two factors are implicated in this, first the " glairy " quality of the mucus itself, and secondly the loss of ciliary action in the bronchial mucous membrane. Florey (1958) has pointed out that the exudate from an inflamed mucous membrane consists not only of mucus produced by glands in the wall and by goblet cells but also of a protein-laden transudate which arises from the increased permeability of the dilated capillaries in the submucosa. A similar process seems likely to occur in asthma. It has already been noted that the exudate in the bronchial lumen is not homogeneous and only partly mucoid in nature. In fact this is in keeping with the allergic basis of the disease. The dilated capillaries lie in oedematous connective tissue just below the basement membrane, and some of the oedema fluid must pass through the mucosa into the bronchial lumen. The presence of this serous fluid exudate closely applied to the bronchial mucosa will affect the ciliary action of the mucosal cells. Hilding (1932) has shown that serous fluid severely curtails the action of cilia, which are much more effective when dealing with pure mucus.

Movement of the exudate will also be retarded by the shedding of the mucosal cells, which was a noticeable feature in this series and was also emphasized by Houston *et al.* (1953). The explanation offered by these authors for the detachment of the mucosal cells was that it is due to muscle spasm and consequent crenation of the mucous membrane with mechanical separation of the epithelium. A more likely factor in this disturbance of the mucosa would be the transudation of oedema fluid across the mucous membrane. There is no situation in human

pathology where contraction of smooth muscle causes detachment of an epithelial surface, but in the skin, e.g., in eczema and the bullous eruptions, disorganization of epithelial architecture by oedema with underlying capillary dilatation, increased capillary permeability, and transudation of protein-laden fluid is commonplace. Disturbances of epithelial continuity are even more likely to occur in the delicate respiratory mucosa following allergic submucosal oedema.

The submucosal oedema may also play some part in the thickening of the bronchial basement membrane. Robb-Smith (1952) has suggested that hyalinization of the basement membrane in the nasal mucosa, following numerous attacks of inflammation, is related to the accumulation of plasma protein and mucopolysaccharide in the inflammatory oedema fluid. Resolution of this oedema results in a condensation of these protein complexes to form "hyaline." A similar mechanism might apply in asthma with continual formation and resolution of submucosal oedema.

It is sometimes claimed that bronchospasm must play some part in the production of an asthmatic attack because of the therapeutic action of adrenaline which relaxes the bronchial smooth muscle. Sheldon and Otis (1951) have shown that an increase in the vital capacity in an asthmatic after adrenaline has been given is not always accompanied by a decrease in air flow resistance as would be expected if adrenaline relaxed the bronchial smooth muscle. They consider that adrenaline acts as a vasoconstrictor on the pulmonary and bronchial arteries, thereby decreasing the quantity of blood in the thorax and allowing more room for air. In some cases air flow resistance is decreased, and here adrenaline may constrict the dilated vessels in the bronchial submucosa, thereby decreasing mucus secretion and submucosal oedema. An explanation for the hypertrophied bronchial musculature may be found in the hypothesis that in cases where ciliary action is defective or absent this muscle plays some part in clearing the bronchial secretions by peristaltic contractions, as suggested by Holden and Ardran (1957) in a bronchographic study using cineradiology. In asthma where ciliary action is so deficient this muscle would undergo a work hypertrophy.

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A CASE OF CEREBRAL MUCORMYCOSIS DIAGNOSED IN LIFE, WITH EVENTUAL RECOVERY

BY

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A case of mucormycosis localized in the brain is reported in a subject aged 18 years who was not suffering from diabetes. As the patient displayed symptoms of cerebral compression he was operated upon and a cystic formation was excised.

Microscopic examination disclosed the presence of hyphae, displaying the characteristics of mucor, in chronic granulation tissue. The mycelia had a tendency to develop in the vessels and to invade them, and a structure very similar to a sporangium could be observed. Some of the mycelia had undergone dystrophic calcification.

The patient has been kept under medical supervision for two and a half years since the operation and appears to have completely recovered.

During the past years numerous cases of human mucormycosis have been diagnosed and its frequent association with diabetes mellitus has been noted. In animals (mice, rats), alloxan-diabetes has been shown to enhance the development and spread of mucor.

The brain is considered to be an infrequent site for mucormycosis. So far 17 instances have been recorded, in 13 of which it appeared to have occurred as a result of spread from the orbital cavities and from the paranasal sinuses (Bauer, Ajello, Adams, and Useda Hernandez, 1955; LeCompte and Meissner, 1947; Foushee and Beck, 1956; Gregory, Golden, and Haymaker, 1943; Gunson and Bowden, 1955; Jackson and Karnauchow, 1957; Kurrein, 1954; Long and Weiss, 1959; Martin, Lukeman, Ranson, and Geppert, 1954; Merriam and Tedeschi, 1957; Muresan, 1956; Paltauf, 1885; Stratemeier, 1950; Torack, 1957; Wolf and Cowen, 1949). Diabetes mellitus was found to be present in 10 of the cases. Post-mortem cultivation of the fungus was successful only in the case of Bauer *et al.* (1955). An exclusively cerebral localization, in the absence of any apparent involvement of other tissues, either local or elsewhere in the body, has not so far been described.

Case Report

The patient, an 18-year-old male hairdresser who lived in a village, had been suffering from headache, giddiness, and vomiting for about three weeks. Slight fever was present. He had involuntary movements of the lower limbs and a progressive decrease of muscular strength in the upper limbs. There were no labyrinthine symptoms, but papillary stasis was found to be present. He was referred to a neuro-surgical department with a tentative diagnosis of brain tumour and found to have signs of compression in the left parietal area. The cerebrospinal fluid was slightly xanthochromic. Papillary stasis and papill-oedema progressively increased and haemorrhagic patches appeared. Laboratory tests yielded no pathological findings. Operation was decided upon.

The suspected area was approached by trepanation and an intracerebral cystic formation, the size of a nut, with turbid, reddish contents, was excised. The peripheral zone of the cyst, of a reddish colour, resembled tumour tissue and the possibility of an astrocytoma was considered.

The post-operative course with penicillin treatment was uneventful and the patient left hospital six weeks after the operation. Insignificant post-operative functional disorders gradually subsided and after six months the patient resumed his occupation. When re-examined two and a half years after the operation, he appeared to have completely recovered and was pursuing his normal vocation.

Microscopical Examination of the Operation Specimen.—The outer part of the specimen was reddish and relatively soft. It enclosed a well-defined cavity displaying a smooth inner surface. Numerous fragments of the tissue surrounding the cavity were examined microscopically. Certain portions consisted of inflammatory granulation tissue containing sparse calcospherites. In these areas, numerous branched, non-septate hyphae of a tubular appearance (Fig. 1)

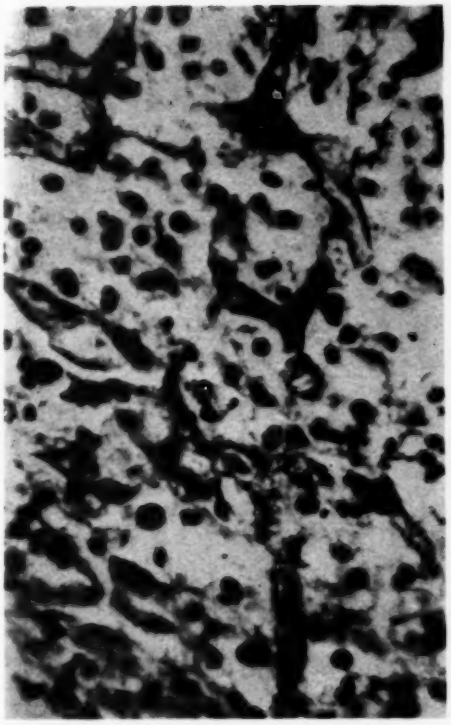

FIG. 1.—Numerous clear branched, non-septate hyphae in non-specific chronic inflammation tissue. Haematoxylin-eosin stain. ×400.

FIG. 2.—The hyphae electively develop around the vessels and display a tendency to invade their walls. Haematoxylin-eosin stain. ×200.

were found. They were often seen to be crowding around the vessels, the walls of which they tended to invade (Fig. 2). Some of the hyphae attained a length of 200 μ (including their branchings). The endings of the branches were irregular or somewhat round, displaying no terminal swellings. A sharply outlined ovular swelling containing roundish structures was found in a single instance. In all probability this was a sporangium (Fig. 3).

As a rule, the fungus hyphae could be uniformly stained by Mayer's haematoxylin and by the Hotchkiss-MacManus method. In some areas, however, the hyphae were seen to be shorter and more condensed, electively staining with haematoxylin as a consequence of dystrophic calcification (Fig. 4). Calcospherites were to be found in the same areas and the inflammatory tissue exhibited a tendency to fibrosis.

Owing to the tentative diagnosis of cerebral tumour the specimen for microscopic examination had been fixed in formol. Thus, inoculations on culture media were impracticable and the fungus could not be classified precisely. Nevertheless it may be inferred from the morphology of the mycelia that the fungus was a member of the family *Mucoraceae*.

Comment

The clinical course and the histopathological findings suggest that in the present case mucormycosis was localized in the inflammatory tissue surrounding a cystic cavity. The fungus probably penetrated into the brain from some other inapparent localization (probably on the nasopharyngeal mucosa or in the upper air passages), whence it was conveyed to the brain via the blood or the lymph. It may be inferred that the cystic cavity in the brain parenchyma followed a focus of softening which was later transformed into a cyst. Very probably the filaments of the fungus, which tend to develop around the vessels and to invade them (see Fig. 2), caused thrombosis of a cerebral vessel resulting in an ischaemic focus and

FIG. 3.—A hypha with an oval ending containing sporulated structures (sporangium). Haematoxylin-eosin stain. ×400.

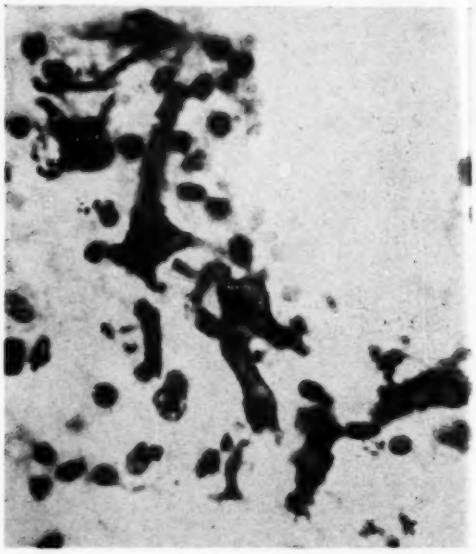

FIG. 4.—Branched hyphae exhibiting areas of dystrophic calcification and irregular margins. Haematoxylin-eosin stain. ×400.

subsequent cystic softening. The fungus continued to develop around the cystic cavity.

The chronicity of the microscopic appearance of the inflammatory process in the area containing fungous hyphae was confirmed by the presence of calcospherites in these areas. Some of the fungous hyphae showed signs of necrobiosis as shown by their calcareous impregnation and by retraction of their margins. It has been shown that calcification of mycelia in the tissues can also occur in cases of chronic histoplasmosis (Muresan, 1956).

A fungous hypha exhibiting a structure similar to the sporangium (Fig. 3) was identified, although not as clearly as in culture media. Mucor sporangia are characterized by the presence of a columnella at the point where the hypha enters the sporangium. This structure was not visible in our microscopical preparations, but, in the opinion of mycologists, the columnella is not seen in all sporangia, even in culture media. Sporangia in the tissues have been observed in other cases of mucormycosis, but only Paltauf (1885) has described them in the nervous system. In other cases of cerebral mucormycosis only filamentous branching mycelia were found.

The interest of this case lies in the fact that it demonstrates the possibility of an elective localization in the brain or other tissues, as the patient recovers after operation, even in the absence of specific antifungal treatment. Also it appears that enhancing conditions (diabetes, leukaemia, antibiotic and cortisone treatment) are not of paramount importance, as has been suggested. Certain microscopic patterns of the development of the fungus in the nerve tissue in this case show that the evolution may be prolonged and that the condition may be well tolerated up to the time when it results in appreciable neurological disorders.

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THE TOTAL WHITE CELL COUNT OF THE BLOOD AS AN INDICATOR OF ACUTE RADIATION DAMAGE AND ITS VALUE DURING THE FIRST FEW HOURS AFTER EXPOSURE

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The total white cell count of the blood is of no value in assessing the degree of acute radiation damage in rats during the first 48 hours after exposure. This is because there is a transient neutrophilia after irradiation which counteracts, to a varying extent, the reduction in numbers of the other white cells. A similar neutrophilia occurs in human patients and there is no reason to expect that the total white cell count over the same period would be of any greater value in man. It is suggested, therefore, that the total white cell count of the blood is not suitable as a method for sorting radiation casualties.

In recent years it has been suggested that routine blood counts are not suitable as controls of chronic radiation exposure (Mole, 1954; Loutit, 1955). However, during the weeks following single large exposures to radiation it is well known that there is a marked reduction in the numbers of the various types of white cells, and detailed examinations of the blood would normally be undertaken in the investigation of small numbers of casualties from excessive exposure to ionizing radiations (Hempelmann, Lisco, and Hoffman, 1952; Guskova and Baisogolov, 1956; Bond, Cronkite, Farr, and Hechter, 1956; Jammet, Mathé, Pendic, Duplan, Maupin, Latarjet, Kalic, Schwarzenberg, Djukic, and Vigne, 1959).

The prognosis of radiation casualties and the choice of treatment depend on the degree of radiation damage and this, in turn, depends on the dose of radiation received. Physical measurements of the dose are, however, unlikely to be available in the first few hours after an emergency involving ionizing radiation, whether it be a peace-time accident or a consequence of nuclear warfare. A clinical assessment of the degree of radiation damage is therefore required. As the number of white cells in the blood some time after a single exposure depends fairly closely on the magnitude of the radiation dose received, it has often been assumed that there will be a similar relationship between dose and effect within the first few hours of exposure. Consequently it has also been assumed that, if laboratory facilities became overloaded because of the number of casualties, it might be possible to use the total white cell count as a means of assessing damage. With this end in view a rapid method of estimating the total white cell count has recently been described and its accuracy considered (Benjamin, 1958; Sanders, Cox, and Sharples, 1958). The accuracy of the method, however, is of no account unless the early changes in white cell numbers do indeed reflect the degree of radiation exposure.

Although the marked leucopenia which occurs some time after irradiation is well recognized, the changes in the leucocyte count occurring during the first 18–24 hours do not appear to be as well known. In particular their implications regarding the use of the total white cell count as an indicator of radiation damage do not seem to have been considered. The object of this investigation has been, therefore, to examine some experimental data from this point of view. It will be shown that the observations indicate that it is not justifiable to rely solely on the total white cell count as an indicator of the degree of exposure to ionizing radiation.

Methods

Heart blood was obtained from rats in which the effects of irradiation on the bone marrow were also being studied. Details of the experimental methods are given in previous reports on other

aspects of the data obtained (Hulse, 1957; 1959a and b). The total white cell counts were performed in the usual manner; two pipettes were filled from each animal and two chambers counted from each pipette, i.e., about 500 cells were counted when dealing with a control. Blood smears were stained by the Jenner-Giemsa method and a differential count performed on 200 cells.

The whole body was irradiated with 240–250 kV x rays and the dose rate was 43 r/min.–68 r/min. A detailed description of the irradiation methods has been given by Corp (1957). One series of animals was given doses ranging from 25 r to 10,000 r and killed at 24 hours after irradiation. A second series was used to study the changes in the white cell count at times ranging from two to 72 hours after irradiation. For this second series three doses of radiation were used: 100 r, a dose much too small to cause acute deaths; 600 r, a dose which gives a mortality of about 50% in the strain of rats used; and 5,000 r, a dose which is uniformly fatal within a few days and is between five and six times that which gives a mortality of 100% from acute radiation damage. Either three or four animals were examined at each dose or time interval, except at 24 hours after 25 r, when six animals were examined. The two series were studied concurrently, and the results for 24 hours after 100 r, 600 r, and 5,000 r refer to the same groups of animals in each case.

Results

Total White Cell Counts.—The total white cell count at 24 hours after various doses of irradiation is shown in Fig. 1. A reduction in the white cell count as the radiation dose increases is apparent, although between 100 r and 1,000 r there was only a threefold change in the mean counts of groups of irradiated animals. Any estimation of the dose of radiation received by an individual from the total white count at 24 hours after exposure is therefore, likely to be very uncertain. In addition it is evident from Fig. 1 that the leucopenia did not depend on dose in a simple uncomplicated manner, the lowest counts being obtained after 800 r and 1,000 r even though the dose range extended to 10,000 r.

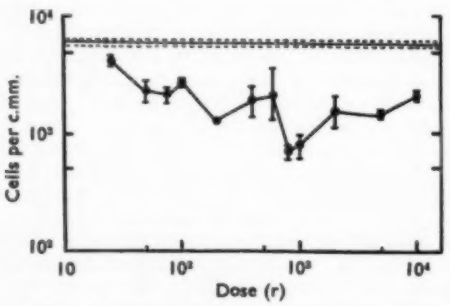

FIG. 1.—Total white cells per c.mm. of blood (means ± standard errors on a logarithmic scale) at 24 hours after irradiation and dose of radiation in roentgens (logarithmic scale). The control value is indicated by a continuous horizontal line and its standard error by interrupted lines above and below it. 200 r point: only two animals were studied at this dose. A standard error has not therefore been given.

The response of the total white cell count at various times after 100 r, 600 r, and 5,000 r is shown in Fig 2. There was a rapid fall in white cells in the first few hours after 100 r and 600 r. The magnitude of the fall was very similar for the two doses, and it was only at times after irradiation greater than 30 hours that differences between these two doses began to be evident. After the larger dose of 5,000 r there was initially a much smaller reduction in the white cell count than after the smaller doses. In fact very little change in numbers from the control level occurred during the first 12 hours, and it was not until 18 to 24 hours after irradiation that the total leucocyte count was depressed as much as it was after 100 r and 600 r. Even at 48 hours the degree of leucopenia after 5,000 r was not significantly greater than that after 600 r.

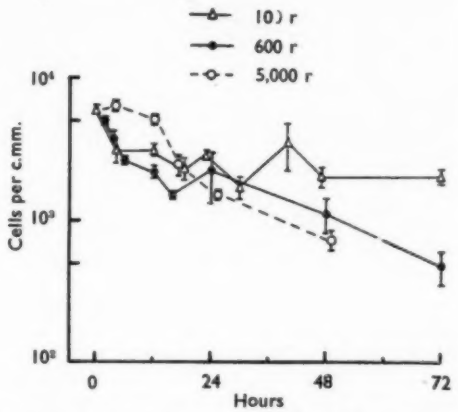

FIG. 2.—Total white cells per c.mm. of blood (means ± standard errors on a logarithmic scale) and time after irradiation in hours for three doses, 100 r, 600 r, and 5,000 r.

There were therefore two findings during the first few hours after irradiation which require explanation: (1) The reduction in the total white cell count after a lethal dose was no greater than that after a much lower sublethal dose, and (2) the total white cell count remained within normal limits for 12 hours after a very high dose.

In order to understand these changes it is necessary to consider how the individual lines of cells respond to irradiation.

Differential Counts.—The data on the various types of white cell are shown in Tables I and II, which correspond to Figs. 1 and 2 respectively. At 24 hours after irradiation there was a progressive decrease with increasing dose in the absolute numbers of eosinophils, lymphocytes, and monocytes (Table I). The responses of the segmented and juvenile neutrophils were, however, rather different, the counts showing no significant difference from the controls over the range of 75 r–600 r but showing real decreases after lower doses (25 r and 50 r) and after higher doses (800 r and 1,000 r). The most striking change, however, occurred after still higher doses when both types of granulocyte increased in numbers and after 10,000 r were well above normal.

TABLE I

ABSOLUTE NUMBERS OF BLOOD LEUCOCYTES AT 24 HOURS AFTER IRRADIATION

Dose of Radiation (r)	Segmented Neutrophils (per c.mm.)	Juvenile Neutrophils (per c.mm.)	Eosinophils (per c.mm.)	Lymphocytes (per c.mm.)	Monocytes (per c.mm.)
25	691 (137)	78 (12)	104 (38)	3,020 (290)	264 (46)
50	350 (73)	62 (2)	70 (42)	1,850 (450)	119 (13)
75	724 (207)	98 (27)	94 (45)	1,140 (180)	124 (30)
100	904 (229)	137 (34)	50 (14)	1,520 (210)	162 (29)
200	735*	125*	18*	332*	67*
400	1,480 (470)	142 (26)	40 (9)	306 (143)	57 (23)
600	1,840 (830)	106 (50)	21 (5)	155 (34)	43 (18)
800	556 (94)	46 (20)	17 (2)	111 (65)	37 (14)
1,000	644 (147)	61 (31)	11 (5)	92 (39)	25†
2,000	1,450 (410)	71 (40)	12 (7)	35 (10)	16 (5)
5,000	1,310 (110)	103 (15)	4†	72 (28)	11 (5)
10,000	1,870 (220)	251 (56)	0	42 (5)	8†
Controls	1,040 (90)	151 (17)	104 (12)	4,450 (260)	275 (22)

Standard errors in parentheses.

* Only two animals were studied at this dose and the standard errors have not therefore been given.

† Standard errors omitted as, in each case, one of the animals gave a zero value.

The responses of the different cell types at various times after irradiation are shown by the data given in Table II. The reduction in the numbers of eosinophils, lymphocytes, and monocytes increased with the passage of time after the two higher doses, and similar but less marked changes occurred after 100 r. During the first 24 hours the segmented and juvenile neutrophils were reduced to not more than half their normal numbers after 100 r but were increased to double normal values after 600 r and to four times normal after 5,000 r. The increases were already evident at two and four hours after irradiation when the first examinations were made, and the maximum recorded response was found at 12 hours after 5,000 r. A neutrophilia may, therefore, occur after irradiation. It was not found after low doses but was present after higher doses, when its duration and its magnitude increased as the dose was increased.

It is thus apparent that the reason why the total white cell count did not change during the first 12 hours after 5,000 r is that the post-irradiation lymphopenia was completely balanced by the neutrophilia. The similarity in the response of the total white count after 100 r and 600 r

TABLE II

ABSOLUTE NUMBERS OF BLOOD LEUCOCYTES AT VARIOUS TIMES AFTER IRRADIATION

Dose of Radiation (r)	Time after Irradiation in Hours	Segmented Neutrophils (per c.mm.)	Juvenile Neutrophils (per c.mm.)	Eosinophils (per c.mm.)	Lymphocytes (per c.mm.)	Monocytes (per c.mm.)
100	4	800 (51)	107 (43)	53 (16)	1,840 (450)	240 (91)
	12	560 (55)	60 (6)	99 (56)	2,280 (190)	82 (38)
	18	591 (179)	58 (25)	72 (16)	1,430 (270)	104 (9)
	24	904 (229)	137 (34)	50 (14)	1,520 (210)	162 (29)
	30	428 (50)	76 (31)	25 (4)	1,110 (220)	49 (15)
	40	793 (238)	89 (14)	67 (27)	1,130 (260)	69 (27)
	48	742 (139)	72 (28)	53 (16)	944 (140)	105 (22)
	72	541 (100)	50 (13)	44 (19)	1,170 (50)	147 (76)
600	2	2,590 (343)	283 (134)	90 (22)	1,780 (130)	277 (55)
	4	2,230 (290)	390 (260)	32 (20)	790 (227)	226 (82)
	6	1,590 (140)	155 (41)	39 (11)	710 (197)	111 (15)
	12	1,220 (290)	179 (95)	66 (21)	602 (440)	66 (41)
	16	1,100 (70)	217 (79)	29 (9)	72 (11)	27 (9)
	24	1,840 (830)	106 (50)	21 (5)	155 (34)	43 (18)
	48	971 (220)	42 (9)	18 (4)	66 (26)	4*
	72	340 (82)	11 (2)	1*	105 (44)	9 (1)
5,000	4	3,690 (110)	990 (71)	41 (8)	1,260 (440)	358 (217)
	12	4,330 (310)	560 (126)	32 (16)	106 (13)	32 (16)
	18	2,140 (430)	332 (26)	13 (2)	52 (24)	15 (8)
	24	1,310 (110)	103 (15)	4	72 (28)	11 (5)
	48	679 (118)	39 (3)	0	13 (1)	3 (1)
Controls		1,040 (90)	151 (17)	104 (12)	4,450 (260)	275 (22)

Standard errors in parentheses. * Standard errors omitted as, in each case, one of the animals gave a zero value.

was also due to the neutrophilia. The lymphopenia was greater after 600 r than after 100 r, but the neutrophilia after 600 r counteracted the decrease in lymphocytes and consequently the total white cell counts for the two doses were very similar. The variations encountered in the data of the response of the neutrophils to the dose (Table I) were also due to the absence of the neutrophilia at low doses and its increasing magnitude and duration at higher doses.

Discussion

In dealing with large numbers of radiation casualties, either civil or military, the aim should be to separate them into three groups, namely, (1) those who will need little if any immediate treatment because they have received only a small dose of radiation; (2) those who have received a dose which may kill and may therefore need the whole gamut of transfusion therapy including marrow grafts (leader, *British Medical Journal*, 1958; Mathé, Jammet, Pendic, Schwarzenberg, Duplan, Maupin, Latarjet, Larrieu, Kalic, and Djukie, 1959); and (3) those who, having received a supralethal dose, will die within a few days in spite of treatment. In the present experimental study the three doses used to obtain the data shown in Fig. 2 and Table II (i.e., 100 r, 600 r, and 5,000 r) produce effects in the rat corresponding to the three categories listed above.

It has been suggested that the total white cell count is the best screening procedure to identify casualties whose radiation injury is severe enough to require medical attention (Benjamin, 1958; Sanders *et al.*, 1958). The results of the present investigation, however, show that the total white cell count in the first few hours after exposure gives no indication of the magnitude of the dose of radiation received. It may be seen from Fig. 2 that during the first 18 hours it is impossible to distinguish between groups of animals receiving small doses of radiation (100 r) and those receiving doses likely to kill (600 r). In addition, the total white cell counts of animals which received a supralethal dose (5,000 r) could, during the first 12 hours, have just as easily come from a group of animals which had received little or no radiation. As the mean white cell count of a group does not allow an assessment of the dose received it is even less likely that a count from an individual would give a reliable indication of the degree of radiation damage he had sustained.

It is not only in rats that the total white cell count soon after irradiation is unreliable as an indicator of acute radiation damage. In the rabbit the results reported by Jacobson, Marks, Simmons, Hagen, and Zirkle (1954) show that the mean total white cell count at 24 hours after irradiation lay within 20% of the control counts for doses ranging from 25 r to 800 r. In man the casualties from a reactor accident in Yugoslavia showed a leucocytosis during the first day after irradiation even though the lymphocyte count was falling (Jammet *et al.*, 1959). The disorganization of the medical services following the dropping of the atom bombs on Hiroshima and Nagasaki (Oughterson and Warren, 1956) prevented study of white cell counts over the period now under discussion.

The inadequacy of the total white cell count as an indicator of radiation damage is due to the increase in the neutrophil count which takes place shortly after irradiation. This relatively transient but nevertheless marked neutrophilia is clearly shown in Table II and in the data given by Jacobson *et al.* (1954) in rabbits and by Jammet *et al.* (1959) in man. The fact that a neutrophilia may occur after irradiation has been known for over 50 years (Aubertin and Beaujard, 1905; Morris, 1905), and the phenomenon has been repeatedly reported after both therapeutic and accidental irradiation in man (Westman, 1921–22; Minot and Spurling, 1924; Goodfellow, 1936; Hempelman, Lisco, and Hoffman, 1952; Court Brown and Abbatt, 1955a; Guskova and Baisogolov, 1956; Bond *et al.*, 1956). It does not, however, seem to have been fully realized that the neutrophilia prevents the total white cell count from being a guide to the degree of radiation damage during the first few hours after exposure.

Ideally the haematological investigation of radiation casualties should include full blood counts, together with platelet counts, reticulocyte counts, and marrow examinations, and the degree of damage should be gauged by the changes occurring in all the cell types. Bond *et al.* (1956) have suggested that even counts of the very radiosensitive lymphocyte are not reliable except after low doses, and the present results (Table II and Hulse, 1959a) support this contention. Using the lymphocyte count, the lowest of the three doses (100 r) can easily be distinguished from the other two doses, but it is not possible to distinguish groups which received doses which might kill (600 r) from those exposed to supralethal doses (5,000 r). During the first 12 hours after irradiation, however, these latter two groups may be distinguished by the different degrees of neutrophilia, the higher dose giving the greater neutrophilia. Thus if the lymphocyte count is

taken in conjunction with the degree of neutrophilia a somewhat more reliable assessment might be made. At later time intervals, however, the neutrophilia subsides and consequently the neutrophil count ceases to assist in assessing damage during the period under consideration.

In circumstances when radiation casualties are too numerous to allow a full haematological examination to be made on each individual some other criterion must be sought to judge the severity of radiation damage. The present data (Table II) suggest that at 24 hours after irradiation the eosinophil count may possibly be of value in spite of the large standard errors, particularly as it may be rapidly performed by a " wet " method. It has been shown that the time of onset after exposure and the severity of post-irradiation nausea and vomiting are dependent upon the dose, i.e., the greater the dose the more severe the symptoms and the sooner they occur (Court Brown and Mahler, 1954; Court Brown and Abbatt, 1955b). It would appear, therefore, that clinical assessments of the severity and time of onset of radiation nausea and vomiting could be used as a screening procedure, but vomiting from other causes, particularly psychogenic vomiting, might make the method unsuitable in practice.

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THE TOXICITY OF RUBBERS AND PLASTICS USED IN TRANSFUSION-GIVING SETS

BY

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The toxicity of different rubbers and plastics used in transfusion-giving sets has been investigated by examining their effects on (*a*) cultures of chick embryo tissues, (*b*) the oxygen uptake of guinea-pig skin tissue cultures, and (*c*) the growth of *Str. pyogenes*.

The results of the laboratory tests have been compared with the incidence of thrombophlebitis after prolonged transfusions through the various materials.

It was found that where the materials inhibited the growth of *Str. pyogenes* they were also toxic to tissue cultures, but that some materials which were toxic to tissue cultures did not inhibit bacterial growth.

The assessments of the relative toxicity of the materials tested by the two tissue culture methods were in agreement. The skin respiration studies, however, gave more information on the early effects of the toxic materials. The relative toxicity of the materials as revealed by these tests could be correlated with the differences in the incidence of thrombophlebitis following intravenous infusions administered through giving-sets assembled with the materials tested.

It is suggested therefore that the toxicity revealed by these tests is of clinical importance, and that tissue culture toxicity tests will prove to be of value in selecting rubbers and plastics for clinical purposes.

It has been shown that the incidence of thrombophlebitis following prolonged intravenous infusions varies with the material of which the transfusion-giving set is made (Handfield-Jones and Lewis, 1952; Medical Research Council, 1957). It is likely that this is due to differences in the liberation of toxic substances from the rubbers and plastics into the infusion fluid. It is therefore clearly desirable to devise a laboratory test by which the relative toxicity of these materials may be assessed. Such a test would have a wide application in medicine generally, as rubbers and plastics are frequently used in close contact with living tissues and for the storage of biological materials.

The suitability of plastics for use in surgery has been assessed by the implantation of samples in laboratory animals (Ingraham, Alexander, and Matson, 1947a, b). While this form of test is necessary to assess the long-term effects of the materials, it has the disadvantage that the non-specific reaction to the trauma of implantation tends to obscure any early toxic effects, so that, unless the toxic reaction is pronounced, the results tend to be equivocal (Barnes, 1953).

By investigation in the intact animal, polymethyl-methacrylate ("kallodent," "perspex") (Beck, Russell, Small, and Graham, 1945) and polyethylene ("polythene") (Ingraham *et al.*, 1947b) have been found to be well tolerated by tissues. "Nylon," which has the advantage that it can be sterilized by autoclaving, has usually been found to be well tolerated as a suture material. These plastics can be obtained in a pure form. Certain other plastics, for instance polyvinyl chloride (P.V.C.), have to be compounded to produce materials with suitable physical properties. Materials of relatively low molecular weight are added as "plasticizers" and other substances as "stabilizers." These added substances may leach out in use, and many of them have been found to be irritating to tissues.

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Plasticized compounds are therefore usually suspect unless it can be demonstrated that they are not tissue irritants.

It has been known for some time that certain rubbers are toxic to living cells. Ranson (1937) reported that rubber was spermicidal, and that rubbers from different sources showed great differences in this respect. Gunz (1948) found rubber to be toxic to cultures of human leukaemic cells, and Lajtha (1952) to cultures of human bone marrow. During the development of a synthetic medium for animal cells in tissue culture, Parker, Morgan, and Morton (1951) observed that chance contact of the synthetic feeding mixtures with the rubber stoppers used to close the culture tubes often killed the cells within 48 hours. Exhaustive extraction with hot and cold solvents reduced the toxicity only slightly. They found that rubbers from different sources showed marked differences in their toxicity, but no sample was completely inert. However, MacDougall (1953) tested silicone rubbers and found them not to be toxic to chick embryo tissue cultures.

In this study the relative toxicity of a series of rubbers and plastics has been assessed by tissue culture methods. In one series of experiments the growth rate of chick embryo tissues in hanging-drop cultures was observed, and in a second series the oxygen uptake of guinea-pig ear skin slices in culture was measured over 24 hours. A similar test has been used for assessing the toxicity of antibiotics (Lawrence, 1959). The materials tested were certain rubbers and plastics on which data were available concerning the incidence of thrombophlebitis associated with their use. Some other materials were also tested for comparative purposes.

As a test for rubber transfusion equipment based on its effects upon the growth of *Str. pyogenes* has been prescribed by British Standard 2463: 1954, most of these materials were also tested for bacterial inhibitory substances.

Materials

The materials investigated were as follows:

Rubber 6107.*—A red natural rubber tubing, referred to as "tubing A" by Handfield-Jones and Lewis (1952), which was manufactured for transfusion purposes from 1944 to 1951.

Rubber 6819.*—A red natural rubber tubing, based on the composition of the tubing referred to as "tubing D" by Handfield-Jones and Lewis (1952). It was manufactured for routine use from 1953 onwards, and inquiries revealed that this was the rubber used during 1956 to make up giving-sets at the North London, South London, Bristol, Birmingham, and Manchester blood transfusion centres. It was therefore the rubber with which the plastic was compared at the centres in England which participated in the trial of plastic giving-sets (Medical Research Council, 1957). The Medical Research Council's Blood Transfusion Research Committee kindly supplied data for the centres using this rubber, not directly obtainable from the published report.

The plastic giving-set† tested was that used in the "thrombophlebitis trial" (Medical Research Council, 1957). A modified plastic giving-set based upon this design has recently been described by Jenkins, Stone, Knowles, Tovey, and Sharpe (1959).

The plastic giving-set provided materials of three types:

Polyvinyl Chloride of Two Different Compositions.—(1) That used for the flexible tubing (referred to below as the P.V.C. tubing), and (2) that used for the stiffer drip tube, drip chamber, and casing of the filter chamber (referred to below as the P.V.C. casing).

Nylon.—The nylon tested was that used in the filter. When it was unexpectedly found to be toxic (see below) the composition of the nylon was discussed with the manufacturer, who thought that there might be residual monomer in the material. The nylon was treated by the manufacturer to remove this substance; the treated material is referred to below as the monomer-free nylon.

Rubber.—The small piece of natural rubber tubing attached to the intravenous needle of the plastic giving-set (referred to below as rubber P.S.).

Tests were also made on silicone rubber D.S.R. 551,‡ the silicone rubber referred to as "colour code yellow" by Wilkinson, Freeman, New, and Noad (1956), who used it for blood transfusions lasting two to four hours, other grades of silicone rubber, and a sample of polytetrafluoroethylene ("fluon").§

Before testing, the rubbers 6107 and 6819 and the silicone rubber were cleaned by the routine process for transfusion tubing (Handfield-Jones and Lewis, 1952); the plastics and rubber P.S. were taken directly from sterile giving-sets as supplied by the manufacturer.

Methods

Chick Embryo Cultures.—Primary outgrowths on coverslip preparations as described by Pomerat (1951) for testing drugs were used to assess the toxicity of the materials. The method was similar to that used by MacDougall (1953) for testing the toxicity of silicone rubber. The cells used were fibroblasts derived from the cardiac ventricles, nerve cells from the spinal cord, and epithelial cells from the intestines of 9–10-day chick embryos. In addition, the mixed

* Manufactured by Messrs. William Warne and Co., Barking, Essex.

† Manufactured by Messrs. Capon Heaton and Co., Stirchley, Birmingham.

‡ Manufactured by the Dunlop Rubber Co., Cambridge Street, Manchester, 1.

§ Manufactured by Imperial Chemical Industries Ltd.

population of cells obtained from 17–20-day chick embryo spleens was used. The tissues were divided into 1 c.mm. fragments and explanted on standard 1¼ in. square coverslips in a medium composed of equal parts of cockerel plasma and chick embryonic extract. The cockerel plasma was freshly prepared and contained no added heparin. The embryonic extract was also freshly prepared from 9–10-day chick embryos, minced by hand and diluted with an equal volume of Tyrode's solution. No antibiotics were used.

In the case of the experimental cultures, the material to be tested was brought close to the explanted tissue before clotting of the medium took place. Approximately 1 c.mm. of material was used. The silicone rubber was sterilized by dry heat; the other materials were autoclaved.

Observations on the amount of growth, the contractility of muscular elements, and the cytological appearance of control and experimental cultures were made after incubation at 38° C. for 18 hours, and thereafter twice daily until 72 hours. Some cultures were fixed in formol-Ringer or acetic alcohol and stained with Ehrlich's haematoxylin and eosin.

Skin Respiration.—Measurement of the respiration of skin slices was made by the technique described by Cruickshank (1954) using a sensitive micro-respirometer, which gives accurate readings of the respiration of skin slices weighing approximately 10 mg. (wet weight). Skin slices were cut free-hand from the dorsum of the guinea-pig ear and suspended in a medium consisting of Krebs-Ringer phosphate (Field, 1948) 60%, glucose, 0.2M 10%, streptomycin, 500 μg./ml. 10%, fresh guinea-pig serum 20%. It will be noted that the amount of serum is less than that previously employed, as higher concentrations of serum have been found to mask minor toxic effects.

The materials were chopped into fine cubes of about 1 c.mm., sterilized by boiling, dried in hot air, and added to the medium, approximately 0.25 g. being immersed in 0.5 ml. of medium in both culture and control cups. In the case of the fine nylon filter which was examined, only about 0.05 g. could be immersed in 0.5 ml. of medium because of its greater bulk. In each experiment the respiration of three control amounts of skin was compared with the respiration of corresponding portions from the same animal in the presence of the test material.

At the end of the experiment the skin slices were fixed in 10% buffered formalin, sections prepared, and these stained with haematoxylin and eosin.

Bacterial Inhibition.—The test described in British Standard 2463:1954, Appendix B, was used, and, at the suggestion of Dr. K. B. Rogers, tests were also made by the following pour-plate technique. A strain of *Str. pyogenes* was grown for 18 hours in 1% glucose-broth and then diluted 1/100 in warm "tryptone" water. Then 0.2 ml. of the diluted culture was added, together with 1.0 ml. of horse blood, to 18.8 ml. of molten heart-infusion agar. The test materials were placed on a layer of heart-infusion agar in a Petri dish. The "seeded" blood agar was then found to give a sandwich plate with the test materials imbedded in the upper layer. The plates were incubated at 37° C. for 18 hours and were photographed within a few hours of their removal from the incubator.

Results

Clinical Findings.—The incidence of thrombophlebitis following intravenous infusions administered through National Blood Transfusion Service giving-sets assembled with rubbers 6107 and 6819 and the Medical Research Council plastic giving-set is shown in Table I. The data

TABLE I

INCIDENCE OF THROMBOPHLEBITIS AFTER INTRAVENOUS INFUSIONS LASTING FROM 13 TO 47.48 HOURS ADMINISTERED THROUGH RUBBER AND PLASTIC GIVING-SETS

Type of Giving-set and Tubing	Total Infusions	Thrombophlebitis	
		No. of Cases	% of Total
*N.B.T.S., rubber 6107	64	42	66
†N.B.T.S., ,, 6819	101	46	46
‡M.R.C., plastic ..	130	33	25

χ^2 calculated with Yates's correction gives the following values for 1 degree of freedom:
Rubber 6107 with 6819 = 5·6, $P<0.02$
,, 6819 ,, plastic = 9·4, $P<0.01$

* Data from Handfield Jones and Lewis (1952), tubing A, added to previously unpublished observations (1953), viz.:

	Total Infusions	No. of Cases
Tubing A { Preliminary survey ..	25	18
Tubing A { Control series ..	19	11
Additional observations	20	13
	64	42

† Data from Medical Research Council thrombophlebitis trial (see text).

‡ Data from Medical Research Council (1957).

concerning the giving-sets assembled with rubber 6107 represent the addition of three consecutive series of observations. These are the "preliminary survey" and "control series" of observations on the incidence of thrombophlebitis associated with "tubing A" made by Handfield-Jones and Lewis (1952) together with a previously unpublished series made by the same authors in 1952–53. The frequency of thrombophlebitis associated with this tubing remained constant ($0.70>P>0.50$), and so it appears reasonable to compare these data with the observations made in the M.R.C. trial in 1956. The infusion period of 13 to 48 hours was chosen for this comparison, as there were insufficient data to make a fair comparison over longer or shorter periods. It will be seen that, in infusions lasting 13–48 hours, rubber 6107 was associated with an

TABLE II
CULTURES OF CHICK EMBRYO TISSUES IN CONTACT WITH VARIOUS MATERIALS

Material Added to Culture	Heart		Spinal Cord		Intestine		Spleen	
	24 Hours	48 Hours	24 Hours	48 Hours	24 Hours	48 Hours	24 Hours	48 Hours
Rubber 6107	−	−	−	−	−	−	− or + away	+ away
,, P.S.	−	− or + away	−	−	−	−	+ away	+ away
P.V.C. casing	−	−	−	−	−	−	+ away	+ away
Rubber 6819	+ away	++ away	+ away	+ away	+ away	+	+ away	++ away
Nylon filter	−	−	−	−	−	−	+ away	+ away
P.V.C. tubing	− or + away	+ away	−	−	−	+	+	++
Silicone rubber D.S.R. 551	++	+++	++	+++	++	+++	++	+++
Nylon filter (monomer-free)	++	+++	++	+++	++	+++	++	+++
None (control) ..	++	+++	++	+++	++	+++	++	+++

− = No growth. + = Slight growth (less than the amount seen in control cultures at 24 hours). ++ = An amount of growth similar to that seen in control cultures at 24 hours. +++ = An amount of growth similar to that seen in control cultures at 48 hours. + Away or ++ away signifies that the growth indicated by the + or ++ sign took place only on the side of the explant remote from the added material.

incidence of thrombophlebitis of 66%, rubber 6819 with 46%, and the M.R.C. plastic set with only 25%.

Chick Embryo Cultures.—The results of 430 cultures are summarized in Table II. Examples of normal growth in a control and an experimental culture are shown in Figs. 1 and 3. Total inhibition of a culture is shown in Fig. 2. Fig. 4 illustrates a somewhat different effect: growth has taken place only at the side of the explant furthest from the test material. This is recorded in Table II as "+away." In assessing intermediate degrees of inhibition the amount of growth in the test cultures was compared with that of the corresponding controls and recorded in a plus notation. This was considered preferable to the recording of growth rates in quarter diameters of the microscopic field as described by Pomerat (1951), as several tissues with varying growth rates were used.

It is obvious from the results shown in Table II that rubber 6107, the P.V.C. casing, the nylon filter, and rubber P.S. were extremely toxic to these cultures and were indistinguishable in this test. Rubber 6819 caused less inhibition of growth, although most of this was away from the rubber. The P.V.C. tubing was less toxic to cultures of spleen than rubber 6819, but on the other tissues which grew more slowly its effect was either the same or slightly more toxic than rubber 6819. The silicone rubber and the monomer-free nylon filter caused no inhibition.

Physiological functions were of little value as an indication of toxicity, e.g., the heart might continue to pulsate for 24 hours although outgrowth had been completely inhibited. The examination of both living and stained cultures, however, showed that, where growth was less than in the corresponding control cultures, various cytological abnormalities such as poor staining reactions, diminished or absent mitoses, and varying numbers of cells with pyknotic nuclei were also present.

By other experiments, not included in Table II, it was shown that tissue from younger embryos was more sensitive to toxic influences than the corresponding tissue from older embryos. Attempts to increase the sensitivity of the tests by reducing the proportion of plasma in the medium were not successful, as sufficient reduction could not be made while maintaining the minimum amount of firmness necessary in the clot.

Repeated autoclaving appeared to reduce the toxicity of rubber 6819, although cells grown in immediate contact were still necrotic and those further away were excessively fatty and far from normal. The toxicity of rubber 6107 remained unchanged after many hours of autoclaving.

The other grades of silicone rubber and the "fluon" which were tested but not shown in Table II were not found to be toxic.

Skin Respiration.—The effects of the various materials on the respiration of guinea-pig ear skin are shown in Table III. Fig. 5 illustrates the relative depression of respiration in relation to time caused by the four principal types of tubing tested. This figure emphasizes the rapid action of the more toxic materials.

As in the chick embryo tissue cultures, rubber 6107 proved extremely toxic, its effect being manifest within two hours. Rubber 6819, the P.V.C. casing, and rubber P.S. did not significantly depress the skin respiration until the second two hours of observation. The nylon filter had no effect in four hours although causing total inhibition of respiration in 24 hours. The P.V.C.

FIG. 1.—Control culture of 10-day chick embryo heart.

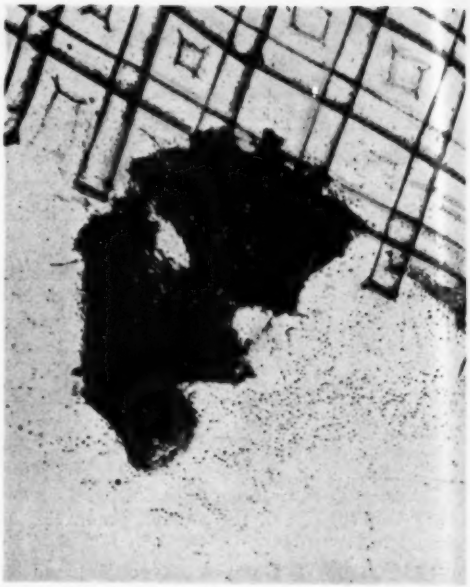

FIG. 2.—Culture of 10-day chick embryo heart in contact with a fragment of the original nylon filter. Note complete inhibition of growth.

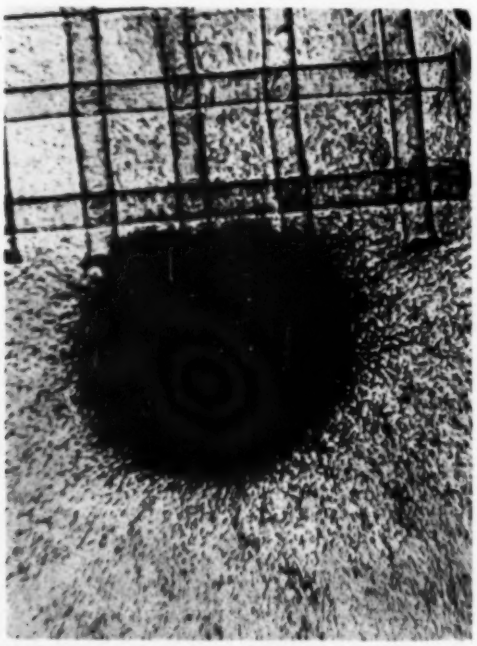

FIG. 3.—Culture of 10-day chick embryo heart in contact with a fragment of the monomer-free nylon filter. Growth is similar in extent to that of the control culture.

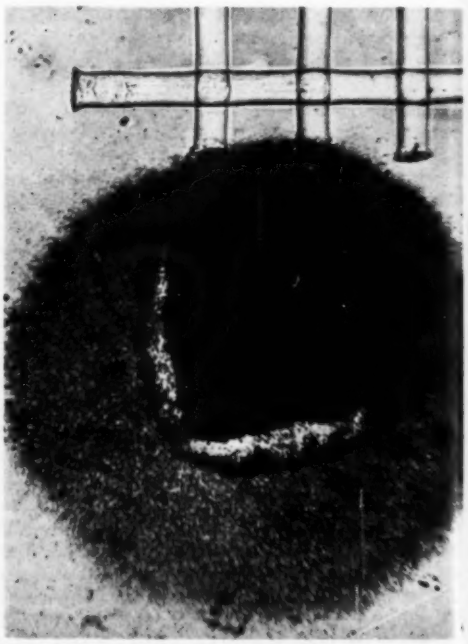

FIG. 4.—Culture of 17-day chick embryo spleen in contact with a fragment of the original nylon filter. Growth is restricted, and has taken place only in the direction away from the nylon filter.

FIGS. 1-4.—*Chick embryo tissues in hanging drop preparations of equal parts of cockerel plasma and chick embryonic extract. Appearances after three days' incubation at 38° C. Stained with Ehrlich's haematoxylin and eosin.* ×30.

TABLE III

THE EFFECTS OF RUBBERS AND PLASTICS ON THE RESPIRATION OF SKIN *IN VITRO*

Material	Time (Hours)	Oxygen Uptake† (μl./mg./hr.)		Difference Test-Control ±S.E.	% Inhibition
		Test	Control		
Rubber 6107	0–2	0·91±0·03	1·11±0·04	−0·20±0·05*	18
	2–4	0·48±0·03	1·22±0·04	−0·74±0·05*	61
	22–24	0·04±0·02	0·89±0·07	−0·85±0·07*	96
Rubber P.S.	0–2	0·96±0·02	1·16±0·11	−0·20±0·11	17
	2–4	0·67±0·02	1·14±0·09	−0·47±0·09*	41
	22–24	0·04±0·01	0·80±0·06	−0·76±0·06*	95
P.V.C. casing	0–2	0·95±0·04	1·05±0·05	−0·10±0·06	10
	2–4	0·98±0·02	1·24±0·05	−0·26±0·05*	21
	22–24	0·06±0·01	0·94±0·04	−0·88±0·04*	94
Rubber 6819	0–2	0·79±0·03	0·86±0·02	−0·07±0·04	8
	2–4	0·71±0·04	0·92±0·00	−0·21±0·04*	23
	22–24	0·14±0·02	0·85±0·02	−0·71±0·03*	84
Nylon filter	0–2	1·08±0·04	0·95±0·03	+0·13±0·05	0
	2–4	1·08±0·03	0·99±0·02	+0·09±0·04	0
	22–24	0·03±0·01	0·78±0·01	−0·75±0·01*	96
P.V.C. tubing	0–2	1·06±0·04	1·18±0·04	−0·12±0·06	10
	2–4	1·02±0·03	1·17±0·04	−0·15±0·05*	13
	22–24	0·56±0·01	0·93±0·10	−0·37±0·01*	40
Silicone rubber (D.S.R. 551)	0–2	1·10±0·03	1·15±0·06	−0·05±0·07	4
	2–4	1·14±0·04	1·17±0·07	−0·03±0·08	3
	22–24	0·93±0·03	0·96±0·07	−0·03±0·08	3
Nylon filter (monomer-free)	0–2	0·97±0·02	1·16±0·08	−0·19±0·08	16
	2–4	1·04±0·03	1·19±0·07	−0·15±0·08	13
	22–24	0·93±0·03	1·05±0·05	−0·12±0·06	11

* The difference between the test mean and the control mean is significant by Student's t test ($P < 0.05$).

† Mean of three experiments ±S.E. mean.

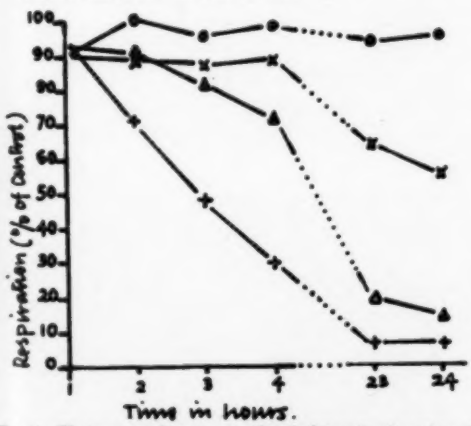

FIG. 5.—The mean respiratory rate at hourly intervals of portions of skin in three respirometers is expressed as a percentage of three control portions. O=Silicone rubber (D.S.R. 551); X=P.V.C. tubing; Δ=rubber 6819; +=rubber 6107.

tubing significantly decreased respiration in four hours, but at 24 hours it had less effect than any of the other materials exhibiting toxicity. The silicone rubber and monomer-free nylon were without effect. The rapid effect of rubber 6107 suggested that the toxic substance might be freely diffusible, and so a further experiment was made to see if it could be removed. The chopped rubber was boiled in repeated changes of distilled water for eight hours: this treatment removed the toxicity. However, similar treatment of rubber 6819 only slightly reduced its toxicity.

Sections prepared after 24 hours' culture showed that the skin remained normal only in the presence of the silicone rubber D.S.R. 551 and the monomer-free nylon filter. The P.V.C. tubing caused minor abnormalities such as occasional patches of necrosis, while the other materials caused severe histological damage.

Bacterial Inhibition.—The results of the tests by the method described in the British Standard 2463:1954 and by the pour-plate technique were in agreement. The pour-plate technique was, however, found to be more sensitive, and the results were more easily photographed.

The effects of the rubbers in a pour-plate are shown in Fig. 6. From rubber 6107 a substance inhibitory to the growth of bacteria has diffused outwards. Beyond the zone of unaltered blood agar the haemolysis is caused by the growth of *Str. pyogenes*; next to the rubber a translucent ring is produced by a haemolytic agent diffusing from the rubber; rubber P.S. is also inhibitory. Rubber 6819 and the silicone rubber are not inhibitory. In Fig. 7 the different effects of the P.V.C.s can be seen; the P.V.C. casing is inhibitory, the P.V.C. tubing is not inhibitory. The original nylon filter was found to be inhibitory, but the monomer-free nylon was not inhibitory.

As it had been found possible to remove the substances toxic to guinea-pig skin from rubber 6107, a further experiment was made to see if the bacterial inhibitory substance could also be removed. Transverse sections of tubing, 2 mm. thick, were boiled in half-hourly changes of distilled water for eight hours. The rings of rubber were removed after one, two, four, and eight hours' treatment. Their effect on bacterial growth can be seen in Fig. 8. The inhibitory substance was gradually removed, but a trace was still present after four hours' boiling in eight changes of water. After eight hours' treatment the rubber was not inhibitory.

Discussion

As information is available concerning the incidence of thrombophlebitis following intravenous infusions given through the rubbers and

FIG. 6

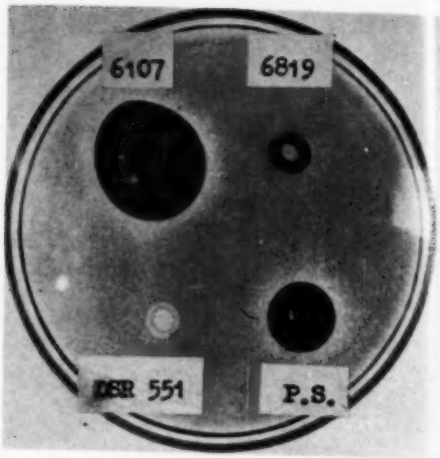

FIG. 6a

FIG. 7

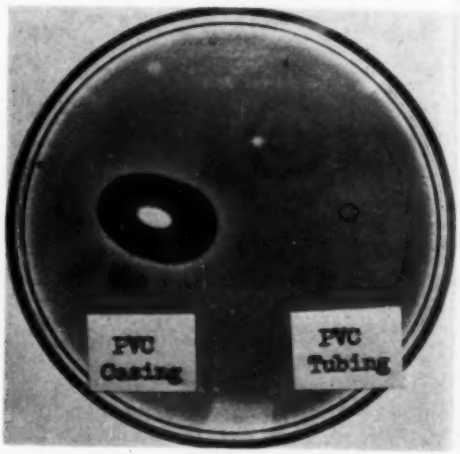

FIG. 7a

FIG. 8

FIG. 8a

FIGS. 6–8.—*The effect of various rubbers and plastics on the growth of Str. pyogenes.*

plastics tested, it is possible to correlate clinical findings with laboratory tests. At the same time a comparison can be made of the effects of these materials on the growth of chick embryo explants, the respiration of guinea-pig skin, and the growth of *Str. pyogenes*.

The two tissue culture tests were in agreement concerning the relative toxicity of rubbers 6107 and 6819. Rubber 6107 was found to be extremely toxic and rubber 6819 less toxic to all the chick embryo tissues and to guinea-pig skin, although, besides differences in the tissues, there were also differences in the culture media and the methods of observation. This assessment, therefore, agrees with the clinical findings, the respective incidence of thrombophlebitis when these rubbers were used being 66% and 46% for infusions lasting 13–48 hours.

Both methods of testing also showed that the silicone rubber and the "monomer-free" nylon were not toxic, and that the small piece of rubber from the plastic set (rubber P.S.) was highly toxic.

The toxicity of the P.V.C. casing and the original nylon filter as assessed by their effects on the respiration of skin at 24 hours agreed with the results of chick embryo tests, and both in this assessment were as toxic as rubber 6107. However, the skin respiration tests gave additional information, as early effects could be observed which enabled distinctions to be made between the materials. The nylon filter had no effect in the first four hours, and the P.V.C. casing, though causing some depression of respiration, had much less effect than rubber 6107. On this basis they are both less toxic than rubber 6107. In comparison with rubber 6819, the P.V.C. casing is of the same order of toxicity, and the nylon filter is less toxic.

As tested on chick embryo cultures and on skin respiration the P.V.C. tubing was less toxic than any of the other materials used in the giving-sets. Its advantage in this respect was most clearly demonstrated by the respiration tests, which showed it to be inferior only to the silicone rubber and monomer-free nylon filter.

It is impossible to be sure what contribution each component of the plastic giving-set makes to its potential total toxic effect in clinical use. It is likely that the relative surface areas exposed to the infusion fluid are of importance. It has been calculated that, of the total surface area in contact with the infusion fluid, the proportionate surface areas exposed are P.V.C. tubing 54%, P.V.C. casing 21%, nylon filter 22%, rubber P.S. 1%, and other parts (coupling needle and Luer adaptor) 3%. It is likely, therefore, that the results *in vitro* are consistent with the reduced incidence of thrombophlebitis when the set is used. It would be of interest to know if the use of a set completely free from toxic effects *in vitro* could achieve a further reduction in the incidence of thrombophlebitis. Other factors, for instance, the *p*H of the infusion fluids, have been postulated as causes of this complication. It is well known that autoclaved dextrose solutions have a *p*H of about 4 (Todd, 1951) and it has been shown by Horvitz, Sachar, and Elman (1943) that the intravenous infusion of amino-acid solutions of *p*H 4.6 into dogs causes more venous damage than the same solutions neutralized to *p*H 7.3. It is, however, uncertain whether acid isotonic dextrose solutions cause venous damage in man (Bolton Carter, Milne, and Whittet, 1952; Page, Raine, and Jones, 1952). As suggested by Page *et al.*, any beneficial effect which occurred in these investigations from adjusting the *p*H of the dextrose solutions was probably obscured by toxic substances liberated from the tubing of the giving-sets used. In any further clinical investigations of this subject it would seem desirable to use giving-sets composed of materials which have been demonstrated to be free of toxic effects *in vitro*.

The bacterial inhibition test appears to be of limited value. All the materials which inhibited the growth of *Str. pyogenes* were found to be toxic. However, rubber 6819 and the P.V.C. tubing which did not inhibit bacterial growth were nevertheless toxic in both tissue culture tests. It is clear, therefore, that this test, though of some value, is an unreliable guide to the selection of suitable rubbers and plastics, and its use will exclude only a proportion of potentially harmful materials.

Prolonged extraction in repeated changes of water removed the toxicity (and also the bacterial inhibitory substance) from rubber 6107, while repeated autoclaving had no effect on its toxicity. In contrast, the toxicity of rubber 6819 was only slightly reduced both by prolonged boiling and by autoclaving. It is doubtful whether this has any practical application for clinical purposes, but the effects of these treatments on the toxicity of tubing must obviously be taken into account in any comparative tests.

The effect of rubber 6107 on skin respiration suggested that the toxic substance was freely diffusible, and it is interesting to note that Barnes (1953) came to the same conclusion from observing its effects after subcutaneous implantation in the rat. He found that it invariably

produced an acute inflammatory reaction with oedema and round cell infiltration, which subsided after 14 days. Samples left in place for three months excited no reaction and were surrounded by a minimum of fibrous tissue. Nevertheless, isotonic saline solutions left in contact with this rubber at room temperature for 72 hours, or autoclaved at 121° C. in contact with it for 30 minutes (ratio of saline to rubber surface, 1 ml.: 8.4 sq. cm.), were without effect when 0.25 ml. was injected intravenously into mice, or when 1.0 ml. was injected intraperitoneally on three successive days. The same solutions when injected intradermally into a rabbit which had been given pontamine blue intravenously to test for increased capillary permeability also had no effect. Rubber 6819 was negative in all these tests.

It was surprising to find that the original nylon filter was toxic. Surgical sutures are not regarded as toxic and this fact was confirmed by subsequent tissue culture tests on a sample. It was suggested by the manufacturer that the presence of free monomer might be the cause of the toxicity, as the nylon monomer contains highly reactive end-amino groups. The non-toxicity of the monomer-free filters subsequently supplied provides confirmation.

The potential toxicity of some rubbers is now fairly widely recognized, but the potential toxicity of the plastics which are tending to replace rubber for many purposes is not always appreciated. Tissue culture tests can give valuable help in the selection of suitable materials for clinical trials. Those which are toxic to tissue cultures would appear to be better avoided. But a negative result in a tissue culture test will not, of course, indicate that the material will be non-reactive when implanted in tissue. Tubes of silicone rubber, for instance, have been found to be not well tolerated in the brain (Dimant, 1955). Several plastics have been found to be carcinogenic in rodents after one to two years' subcutaneous implantation (Oppenheimer, Oppenheimer, Danishefsky, Stout, and Eirich, 1955).

It is probable that tissue culture tests will be chiefly of value in assessing the suitability of rubbers and plastics for use as catheters, endotracheal tubes, etc., and for apparatus used in contact with fluids which are infused, as in blood transfusion giving-sets, heart-lung machines, etc. It is possible that tissue culture tests might show variation in the toxicity of the plastic containers used for storing blood. Some of these have had deleterious effects on the red cells stored in them (Wall, Buckley, and Doan, 1953; Strumia, Colwell, and Ellenberger, 1955). These tests will probably also be found to be of value in testing batches of the selected variety of plastic (or rubber) before it is used. This would be of particular importance when the exact composition of the material is unknown and possibly subject to variation. Detailed information on the composition of rubbers and plastics is unfortunately not often available for publication, and, in some instances, the manufacturers of an article may themselves not know the exact composition of the compounds supplied to them by the manufacturers of the basic material.

We should like to thank Dr. R. P. C. Handfield-Jones for permission to quote unpublished observations made jointly with one of us (H. B. M. L.); Mr. J. E. Dowse, A.I.M.L.T., for assistance with the bacterial inhibition tests; and Mr. A. Boot, of Messrs. Capon Heaton & Co., for his co-operation. We are also indebted to Miss A. H. Ogilvie and Mr. A. S. Pyper for technical assistance.

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PENICILLIN ANTIBODY

BY

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A serum which specifically agglutinated penicillin-coated erythrocytes is described. The active constituent had the characteristics of an antibody. Its laboratory and clinical significance is discussed.

The antibiotics chloramphenicol (Glazko, Wolf, and Dill, 1949), penicillin, streptomycin, aureomycin, and terramycin (Seigneurin, Achard, and Magnin, 1955) are readily adsorbed to erythrocytes. Seigneurin, Magnin, and Fils (1958) observed that adsorbed penicillin, streptomycin, aureomycin, and erythromycin resist elution, yet staphylococci were killed by washed erythrocytes which had been exposed to these antibiotics. Seigneurin *et al.* presumed that this was due to slow release of the antibiotics and considered that erythrocytes thus play an important part in the successful outcome of antibiotic therapy.

The discovery of a circulating antibody to penicillin (Ley, Harris, Brinkley, Liles, Jack, and Cahan, 1958b), which specifically agglutinated erythrocytes coated with penicillin, has introduced a relatively simple means of studying the immunological aspects of antibiotic therapy. The antibody agglutinated erythrocytes treated with penicillins G, O, and K, or with a penicillin derivative, synnematin B, but not those treated with several other antibiotics. Prior exposure of the serum to penicillin inhibited the antibody. Penicillin which had been inactivated with penicillinase failed to sensitize erythrocytes to the antibody; penicillin-coated erythrocytes, however, were agglutinated by the antibody even after they had been exposed to penicillinase for four hours. The antibody was present in the sera of 25 out of almost 2,000 normal blood donors (1.25%) (Ley, cited by Greenwalt, 1958), in 41 out of 413 hospital patients (9.9%), and in seven out of 21 persons (33.3%) known to be hypersensitive to penicillin (Ley, Cahan, and Mayer, 1958a). In some sera the antibody was demonstrated by direct agglutination and in others by the antiglobulin test. All persons whose sera contained the antibody had received or were receiving penicillin; only a minority of these had demonstrated clinical sensitivity to penicillin

Characteristics of a Penicillin Antibody

This paper briefly describes some characteristics of an antibody to penicillin found during a survey of the sera of hospital patients in Poona. The methods adopted were those of Ley *et al.* (1958b). The antibody was the only strong one out of five examples found in the sera of 187 patients (2.7%). It agglutinated red cells coated with penicillin but not untreated cells or those coated with streptomycin.

The antibody was active in both slide and tube tests. Agglutination was delicate; vigorously rocking the slides or shaking the tubes caused the red cells to disaggregate. Red cells suspended in isotonic saline, tested in tubes against doubling dilutions of serum, were agglutinated to a titre of 16. When AB serum was substituted for saline there was no significant increase in titre; cells suspended in albumin were not agglutinated.

The agglutinin acted as strongly at room temperature as at 37 or 4° C. It was specifically inhibited by previous exposure of the serum to penicillin for 30 minutes. A concentration of at least 125,000 units of sodium penicillin G per millilitre of isotonic saline was necessary; this is over 20 times the concentration which was required by Ley *et al.* (1958b) to inhibit one of their antibodies. When agglutinates were carefully pipetted on to a slide, the addition of a drop of penicillin solution caused them to break up; they were unaffected by streptomycin or saline solution.

The serum, when undiluted or diluted 1 in 2, produced large loose agglutinates which looked

more like those formed by strong anti-Le^a sera than like heavy rouleaux; perhaps this type of agglutination occurs when the reactive antigen is passively attached to the red cells. Rouleaux formation was excluded because untreated cells or those treated with streptomycin remained evenly dispersed when tested under the same conditions.

The agglutinin was adsorbed by penicillin-coated erythrocytes and eluted; the eluates specifically agglutinated "penicillinized" cells. The serum became inactive after incubation with the proteolytic enzyme trypsin for two hours at 37° C.; the active component is therefore a protein.

The patient from whom the serum was obtained had received 1 million units of penicillin twice a day for 10 days three months previously. He was clearly not hypersensitive to penicillin, because he had been given a penicillin injection just before the antibody was reported to the clinicians. A second sample of serum collected two weeks later showed no change in antibody titre.

Discussion

The serum protein described has many of the characteristics of an antibody. Although the history is suggestive, there is really no evidence that it is of immune origin; on the other hand its capacity to act as well at low temperatures as at 37° C., its non-potentiation by AB serum, its inactivity in albumin, and its failure to increase in titre two weeks after the appropriate stimulus indicate that it might not be an immune antibody. It is thus uncertain that it is an antibody in the strict sense; however, the term is justified in that it is now applied (Wilson and Miles, 1955) to a certain type of serum protein which reacts specifically with antigen without insistence that its synthesis is dependent on introduction of the antigen.

Agglutinins for penicillin-coated erythrocytes are of laboratory, and possibly clinical, significance. The widespread practice of storing panels of fully grouped erythrocytes in ACD-penicillin-streptomycin mixtures may seriously hinder the identification of blood group antibodies. Penicillin should no longer be a component of erythrocyte preservative solutions.

A more dangerous practice, fortunately uncommon, is the addition of penicillin to blood stored for transfusion. The author knows of two blood banks in which this is a routine measure. These institutions have experienced some haemolytic transfusion reactions which they could not ascribe to any known blood group antibody; in the light of recent developments it is possible that anti-penicillin antibodies were responsible. It may be stated that it is unwise to add penicillin to banked blood for a different reason. The most dangerous bacterial contaminants of stored blood are certain strains of *E. coli* and *Ps. aeruginosa* which multiply readily at blood bank temperature (James and Joan Stokes, 1957); these organisms are insensitive to penicillin.

The clinical significance of anti-penicillin antibody is obscure. It appears from the author's case and the experience of Ley and his colleagues that the presence of antibody is not necessarily associated with clinical sensitivity to penicillin; nevertheless Ley *et al.* (1958a) found a higher incidence of antibody among persons sensitive to penicillin than among others. It is possible that the antibody responsible for clinical sensitivity is different to the agglutinin for penicillin-coated erythrocytes.

Ley *et al.* (1958a) observed, in a person whose serum contained the agglutinin, that the survival time of transfused erythrocytes previously exposed to high concentrations of penicillin (10–50 mg./ml.) was greatly decreased, whereas it was almost normal in a control. After they gave heavy doses of penicillin (18 million units a day) to a patient with these antibodies in her serum, the erythrocytes developed a positive antiglobulin test and there was a significant fall in the haematocrit. Such doses are unusual; the occurrence of antibody-induced haemolytic anaemia consequent upon penicillin therapy would thus be a remote possibility.

The antibacterial effect of penicillin *in vitro* was not impaired in the presence of anti-penicillin serum (Ley *et al.*, 1958a); moreover, serum penicillin levels, after the antibiotic was administered to persons who had made the antibody, were the same as in controls. Thus, although the haemagglutinating capacity of the antibody was inhibited by penicillin, the antibody did not impair the effect of penicillin on bacteria. This might have been due to excess penicillin; in view of the high concentrations of penicillin required to inhibit, a more reasonable explanation is that the complex of penicillin and antibody has as great an antibacterial effect as penicillin alone.

Hazards arising from acquired bacterial insensitivity to penicillin, those of clinical hypersensitivity to penicillin, and those possibly associated with agglutinins for penicillin-coated

erythrocytes make it imperative that the indiscriminate administration of penicillin should be avoided.

I am indebted to Lieutenant-Colonel S. N. Chak for a generous supply of serum.

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THE BACTERIOLOGY OF URINARY INFECTIONS IN PARAPLEGIA

BY

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Two hundred and forty-seven strains of micro-organisms were isolated from the urinary tract of 121 patients with neurological complaints leading to paraplegia. The commonest infecting organism was *E. coli* and the commonest type of Proteus was *P. mirabilis*. Infections with more than one type of organism were present in 37% of urine specimens, and in such cases the incidence of *E. coli* was lower and that of *Strep. faecalis* higher than the incidences in infections with only one type of organism.

No evidence was found which might suggest that the various bacterial species encountered showed either an undue degree of synergism or of mutual antagonism.

Coliforms were the commonest primary pathogens, but *Strep. faecalis* and Proteus species occurred with the same frequency as the coliforms when they were considered as secondary invaders.

The variance between the incidences of various species as reported by different workers is considered to be due to the different illnesses and conditions which predispose to urinary tract infection. It appears that infections in the urinary tract by more than one type of organism at a time occur more frequently in those patients with the more chronic predisposing causes. (Some disparity in findings may also result from the differing taxonomic methods employed by different workers, but this is probably insufficiently important to account for the largest differences.)

In the course of routine examination of the urines of paraplegic patients in this hospital the high incidence of infected urines containing more than one type of organism was noted. It was decided to investigate paraplegic urines with specific reference to the frequency of various bacterial species, the possibility of the bacteria in multiple infections exerting mutual antagonism or synergism, and the relative importance of organisms as primary pathogens and as secondary invaders of the urinary tract. It is proposed to discuss the results against a review of the findings of certain other workers in other branches of medical and surgical practice.

Material and Methods

The number of patients with urinary tract infections investigated in one calendar year was 121. All of the patients had paraplegia in some form or another and in no case could it be shown that the infection began suddenly as, for example, in an acute pyelonephritis. Whenever possible specimens of urine were obtained for culture from women by catheterization and from men by catheterization or by the "mid-stream" technique, but many patients at one time or another were on some form of bladder drainage, and this was then used as the means of obtaining the specimen. The organisms were isolated primarily on McConkey's bile salt lactose agar and blood agar. Species were identified by morphological and biochemical methods. The coliforms, i.e., all Gram-negative lactose-fermenting bacilli, were classified on the basis of indole production after two and seven days' growth in peptone water, the utilization of citrate (Koser's medium), the methyl-red reaction after growth in glucose-phosphate medium, and the Voges-Proskauer reaction using creatine and sodium hydroxide to hasten the reaction.

The Proteus group was classified according to the method of Cook (1948). Christensen's urea medium and lead acetate agar were used for the detection of species producing urease and H_2S respectively.

Coagulase-positive staphylococci were so designated after slide tests and negative strains subjected to a tube test.

β-Haemolytic streptococci were so designated on cellular and colonial morphology, and by β-haemolysis on blood agar.

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Streptococcus faecalis was differentiated from other streptococci by its ability to withstand a temperature of 60° C. for 30 minutes.

The actual media and techniques used were as described by Mackie and McCartney (1953).

In this paper the phrase "primary pathogens" is taken to include the organisms isolated in the initial specimen of urine received from a patient and also those isolated after a period in which one or more urine specimens had proved sterile. All other organisms are regarded as secondary invaders. Although many patients were undergoing antibacterial therapy, no attempt is made here to separate secondary infection from superinfection.

Results

Frequency of Various Bacterial Species.—The frequency with which the various bacterial species were found is as follows:

Bacterial Flora in All Urines.—The total number of strains of micro-organisms isolated at varying times in the study period from the 121 patients was 247, and these were recovered from a total of 181 specimens of urine. Of the patients, 49 were men and 72 women (see Table I).

TABLE I
NUMBER OF PATIENTS AND URINE SAMPLES INCLUDED IN SERIES

No. of Patients	Sex		No. of Infected Urine Samples Examined			No. of Strains of Micro-organisms
	M	F	Total	Single Infection	Multiple Infections	
121	49 (41%)	72 (59%)	181	114 (63%)	67 (37%)	247

TABLE II
INCIDENCE OF VARIOUS BACTERIAL SPECIES FOUND IN URINE IN PATIENTS WITH PARAPLEGIA

Organism	Overall Incidence	Incidence in Single Infections	Incidence in Multiple Infections
	No.	No.	No.
Coliforms ..	92 (36%)	55 (48%)	37 (28%)
Strep. faecalis ..	54 (23%)	14 (12%)	40 (30%)
Proteus (all types)	53 (21%)	27 (24%)	26 (20%)
Ps. pyocyanea ..	24 (10%)	8 (7%)	16 (12%)
Miscellaneous ..	24 (10%)	10 (9%)	14 (10%)
Total ..	247 (100%)	114 (100%)	133 (100%)

The incidence of the various bacterial strains isolated is given in Table II. As can be seen, the descending order of frequency is: coliforms, *Strep. faecalis*, Proteus, *Ps. pyocyanea*, and lastly a group of miscellaneous strains.

The coliforms were found to be divided as follows: *Bact. coli* 77%, *Bact. intermedium* 13%, and *Bact. aerogenes* 10%; the Proteus species were as follows: *P. mirabilis* 85%, *P. vulgaris* 10%, and *P. morgani* 5%.

Of the miscellaneous group, comprising 24 strains, seven were coagulase-positive staphylococci, 12 coagulase-negative staphylococci, three β-haemolytic streptococci, one a candida, and one a corynebacterium.

Bacterial Flora in Urines Containing Only One Type of Organism.—A single infecting organism was found in 90 patients, and from them 114 strains were isolated at different times. The incidence of the bacterial strains in these patients was: coliforms, Proteus, *Strep. faecalis*, the miscellaneous group, and *Ps. pyocyanea*, again in descending order of frequency. The actual frequencies found are shown in Table II. It will be seen that the coliform group formed by far the commonest single infecting organism, with the Proteus group next in order of frequency.

Bacterial Flora in Urines Containing More Than One Type of Organism.—A total of 133 strains was obtained from 67 specimens of urine containing more than one type of organism. The frequency of the various organisms found in multiple infections of the urinary tract was, in descending order: *Strep. faecalis*, coliforms, Proteus, *Ps. pyocyanea*, and the miscellaneous group. The incidence of coliforms was decreased considerably, while that of *Strep. faecalis* was increased. From the figures given in Table II it can be calculated that these changes in incidence are statistically significant ($P = <0.01$ in both cases). It will also be noticed that Proteus species have retained the same frequency which they possessed in single infections.

Analysis of Frequency of Association Between Various Species.—It is possible to show mutual antagonism between certain species *in vitro*; for example, that between certain strains of Pseudomonas and Proteus. Similarly, it is possible to show a "synergism" between species, e.g., the "satellitism" of *Haemophilus influenzae* around colonies of staphylococci. It was considered possible that similar antagonisms or synergism might occur *in vivo*.

It was decided that this problem could best be considered by analysing the results in the following manner. One strain at a time was selected for reference, and the number of other strains that were found in association with it was noted. Expressing the numbers so found as percentages of the number in the reference strain enabled direct comparisons to be made. The

TABLE III
FREQUENCY OF ASSOCIATION OF DIFFERENT SPECIES IN MULTIPLE INFECTIONS

Percentage of Other Strains Associated with:	Coliforms	*Strep. faecalis*	Proteus	*Ps. pyocyanea*
Coliforms	—	52	24	13
Strep. faecalis ..	53	—	23	20
Proteus	38	38	—	8
Ps. pyocyanea ..	31	50	13	—

results are given in Table III. By way of example it can be seen that, of all the strains found in association with the coliforms, 24% were Proteus species.

From inspection of Table III it would appear that the association between coliforms and *Ps. pyocyanea* is less frequent (31%) than the association between coliforms and *Strep. faecalis* (50%), for example. Also, when the coliforms are considered as the reference strain, it is seen that only 13% of organisms associated with them were *Ps. pyocyanea* as against 24% for Proteus. A similar apparent antagonism between Proteus and *Ps. pyocyanea* seems possible, as does an apparent synergism between *Strep. faecalis* and *Ps. pyocyanea.*

From the figures in the accompanying tables it is possible to calculate the expected frequencies of association between each type of organism. When this is done it is found that the expected frequencies agree remarkably closely with the observed frequencies, and that therefore a significantly abnormal amount of association between the species and the apparent antagonisms and synergisms discussed above did not exist.

Relative Importance of Organisms as Primary Pathogens and as Secondary Invaders.—The coliforms were found to be the commonest primary invader, with *Strep. faecalis* and Proteus next equal in importance. As secondary invaders, however, the three organisms occurred in almost equal frequency (Table IV).

TABLE IV
PERCENTAGE FREQUENCY OF VARIOUS SPECIES AS PRIMARY AND SECONDARY INVADERS

	Coliforms	*Strep. faecalis*	Proteus	*Ps. pyocyanea*	Miscellaneous
Primary invaders	42	15	18	11	14
Secondary invaders	29	26	29	10	6

Discussion

In this paper no attempt is made to differentiate between true infections and "benign bacteriuria," since any particular organism occurring in a benign bacteriuria is, theoretically at least, in a position to initiate a true infection.

The observed frequency of occurrence of coliforms as the commonest infecting organisms

TABLE V
PERCENTAGE FREQUENCY OF VARIOUS ORGANISMS IN URINE AS FOUND BY VARIOUS AUTHORS

Author	Coliforms		*Strep. faecalis*		Proteus		*Ps. pyocyanea*		Staphylococci		Streptococci		Others		Single Infections	Multiple Infections	Nature of Cases
	S	M	S	M	S	M	S	M	S	M	S	M	S	M			
Hill *et al.* (1929)	90		—		3		—		—		—		7		—	—	Non-neurological
Rantz (1942)	78	22	7		2		—		29		30		6		62	38	Pyelonephritis and cystitis
Badal *et al.* (1944)	27		9		24		2		14		15		9		—	—	Spinal cord injuries
Gould (1951)	68	81	7	56	2	17	1	2	18	28	1	28	—		—	—	? nature of cases
Gould *et al.* (1952)	51		5		19		14		—		—		11		—	—	Non-neurological
Marshall (1952)	37		9		8		20		5		5		16		83	17	Prostatectomy
Alesbury (1952)	23		35		—		—		5		37		—		—	—	Late pregnancy and puerperium
Yow* (1952)	70		—		9		15		—		—		10		—	—	In 1948 ? nature
	36		—		35		32		—		—		36		—	—	,, 1950 ? cases
Rhoads *et al.* (1952)	50		22		3		15		7		—		3		79	21	? nature of cases
Barnard *et al.* (1953)	41		5		3		0		20		7		24		—	—	Diabetic women
Ghormley *et al.* (1954)	19		11		—		4		45		14		7		50	50	Chronic prostatitis
Sanford *et al.* (1956)	51	35	9	10	20	20	5	7	5	20	—	—	10	8	80	20	? nature of cases
Scott (1959) (this series)	48	28	12	30	24	20	7	12	—		—		9	10	63	37	Paraplegia

* The figures here are expressed as a percentage of the total number of urines cultured.

S = Single infections. M = Multiple infections. (Where S and M are not specified in dividually the single figure refers to the total incidence of that particular strain.)

is to be expected. Similarly the increased frequency of isolation of *Strep. faecalis*, Proteus, and *Ps. pyocyanea* in multiple infections might be anticipated. These findings are, nevertheless, at variance with those of certain other workers (Table V). Gould (1951), for instance, in a series of 658 cases of urinary tract infection, found *Bact. coli communis* in 68% of single infections, whereas in multiple infections the figure rose to 84%. The patients in his series were in a general hospital, and it seems probable that most were not neurological cases and not of the same order of chronicity as in the paraplegic patients in this series, and that the figures were based on the results of the initial urinary specimen examined in each case. Other series show varying figures for non-neurological cases. Marshall (1952) isolated coliforms in 38% of prostatectomy cases, whilst Hill, Seidman, Stadnichenko, and Ellis (1929) isolated Escherichia in 50% and Aerobacter in 39.5% of non-neurological cases, i.e., a total of 89.5% for coliforms as a whole, but this latter investigation was in the pre-antibiotic era. On the other hand, Alesbury (1952) isolated coliforms in only 23% of cases in late pregnancy and in the puerperium. In his series coliforms were third in succession to streptococci (throat type, 37%) and enterococci (35%). Rantz (1942) reported an incidence of 78% of coliforms in single infections, a figure which fell to 22% for multiple infections. His work shows the largest disparity with this series, but was confined to patients with pyelonephritis and cystitis with or without septicaemia (Table V).

Strep. faecalis is found more often in the urine as a secondary invader than as a primary pathogen. There is general agreement upon this in the findings of other workers (Table V). There is general agreement also that this organism is less commonly found than the coliforms. A notable exception is the series of Alesbury (1952), who found an incidence of 35% for *Strep. faecalis*; and it is probably very significant that his findings are in women in late pregnancy and the puerperium. That this is probably not due to a sex difference is shown by the work of Barnard, Story, and Root (1953), who found an incidence for *Strep. faecalis* of only 5% in 52 diabetic women.

There are fairly wide differences in the frequencies given by various workers for the Proteus group, and it is difficult to draw any conclusions concerning it. However, it does seem possible to make the generalization that this group is found with increased frequency in those patients with the more chronic conditions—a generalization that can, in fact, be applied to all types of organisms other than the coliforms.

From Table V it appears that there is a slight correlation between the incidence of multiple infections and the duration of the predisposing causes of the infections. The lowest incidence of multiple infections (17%) is seen in Marshall's (1952) series in which the patients were undergoing prostatectomy. In this series 67 out of the 181 infected urine specimens contained more than one type of organism (27%), whereas in the series of Ghormley, Cook, and Needham (1954) an incidence of 50% for multiple infections was found in a series of cases of chronic prostatitis, in which, it is interesting to note, staphylococci (of all types) were frequently encountered (45%).

As stated earlier, a significant degree of association between the various bacterial species was not shown to exist. This does not preclude such an association being found in a much larger series.

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THE BIOLOGICAL SIGNIFICANCE OF THE ANOMALOUS SERUM AND URINARY PROTEINS OF MYELOMATOSIS, LYMPHOMA, AND OTHER CONDITIONS

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The frequent occurrence of protein anomalies in patients with myelomatosis has long been recognized. Bence Jones reported on the unusual urinary protein of a patient with this disease over a hundred years ago (Bence Jones, 1848). Hyperproteinaemia in myelomatosis was recorded somewhat later (Ellinger, 1899). Subsequently, electrophoretic studies have shown that the hyperproteinaemia is due to the presence in serum of a protein or proteins apparently absent from normal serum.

In the past decade, however, it has become increasingly evident that anomalous protein components in serum or urine are not restricted to myelomatosis but occur in various other conditions. Such components have been the subject of many investigations, and it is the aim of this review to summarize current views on the nature and significance of these proteins.

Occurrence of Anomalous Serum and Urinary Proteins

For the present purpose, anomalous proteins are defined as those which appear as discrete abnormal components in the electrophoretic patterns obtained with conventional techniques, viz., boundary electrophoresis or zone electrophoresis on paper. A number of names, based either on the circumstances in which they are found or on their chemical properties, have been given to such proteins occurring in serum. Thus those found in myelomatosis are termed myeloma proteins, those with high molecular weights are termed macroglobulins, whilst those which precipitate on cooling are termed cryoglobulins. The use of such terms, however, tends to obscure the many affinities of these proteins. Anomalous urinary proteins comprise Bence Jones proteins.

Conditions in which anomalous proteins have been found fall into three groups, namely neoplastic disease, idiopathic haemolytic disease, and miscellaneous conditions.

Neoplastic Disease.—By far the commonest conditions associated with anomalous proteins are myelomatosis and lymphoma. About 80% of patients with myelomatosis have anomalous electrophoretic components in their sera ; a smaller proportion of cases excrete Bence Jones protein in the urine (for references see Owen and Rider, 1957). Lymphoma (using this as a generic term to include conditions designated more precisely lymphosarcoma, reticulum cell sarcoma, or Hodgkin's disease) is less frequently associated with anomalous serum components, although many instances have been recorded (e.g., Mackay, Taft, and Woods, 1957 ; Azar, Hill, and Osserman, 1957 ; Quattrin, Dini, and Piccoli, 1956 ; Mackay, 1959).* Bence Jones proteinuria also occurs in lymphoma, though it is less common than anomalous serum components (Mandema, van der Schaaf, and Huisman, 1955 ; Jim and Steinkamp, 1956 ; Mackay *et al.*, 1957). Some patients with anomalous serum proteins present clinical and histological features intermediate between those of myelomatosis and malignant lymphoma (Mandema, 1954 ; Osserman and Gellhorn, 1955 ; Regniers, Wieme, Wunderly, and Burtin, 1956 ; Azar *et al.*, 1957 ; Motulsky, Eriksen, Volwiler, and Donohue, 1958 ; Owen, Pitney, and O'Dea, 1959). Indeed the two conditions appear very closely related.

Occasionally, anomalous serum components have been recorded in lymphatic leukaemia (Rundles, Coonrad, and Arends, 1954), in

*Some authors have regarded lymphoma with anomalous serum components as a condition distinct from lymphoma without such components, calling the former condition "macroglobulinaemia" (Waldenström, 1944) on the grounds that the anomalous serum component is usually a macroglobulin. There would seem to be, however, no more justification for this than for regarding myelomatosis with myeloma serum proteins as distinct from myelomatosis without these proteins.

monocytic leukaemia (Brown, Read, Wiseman, and France, 1948), and in chronic myeloid leukaemia (Brown *et al.*, 1948). Such components have also been found in patients with myelofibrosis (Lucey, Leigh, Hoch, Marrack, Johns, Kekwick, and Holiday, 1950; Videbaek, 1955; Owen *et al.*, 1959) though there was evidence in these cases of concomitant myelomatosis or lymphoma.

In a few instances anomalous serum components have been found in patients with malignant tumours in various sites (Schaub, 1953; Kanzow, 1954; Laurell, Laurell, and Waldenström, 1957; Waldenström, 1958; Kappeler, Krebs, and Riva, 1958; Owen *et al.*, 1959). However, in some, but not all, of these cases there was evidence of a co-existing lymphoma. Bence Jones proteinuria has been found in cases of bronchial carcinoma (Copeland, 1931; Hughes, 1954).

Idiopathic Haemolytic Disease.—Christenson and Dacie (1957) have noted the frequent association of anomalous serum electrophoretic components in patients with idiopathic haemolytic anaemia of the cold-agglutinin type, and similar cases have been reported by others (Sonnet, Louis, and Heremans, 1955; Fudenberg and Kunkel, 1957; Fudenberg, Barry, and Dameshek, 1958; Owen *et al.*, 1959). The association of anomalous serum components with haemolytic anaemia has also been found in patients with lymphoma (Craig, Waterhouse, and Young, 1952; Buffa and Rappaport, 1957; Christenson and Dacie, 1957). In contrast, anomalous serum components were not found in haemolytic anaemia of the warm-agglutinin type (Christenson and Dacie, 1957; Charbonnier and Dausset, 1953).

Miscellaneous.—There are a few isolated reports of anomalous serum components in various other conditions. Of particular interest is the finding (Laurell *et al.*, 1957) of such a component in the serum of a blood donor who showed no other physical or laboratory signs of disease. Other reports have concerned patients* with obscure anaemia (Flynn, 1954; Pitney, O'Sullivan, and Owen, 1958); hepatitis (Wieme, 1953); chronic urticaria (Motulsky *et al.*, 1958); granulomatous lung disease (Motulsky *et al.*, 1958); and cases in which a definite diagnosis was not established (Martin and Close, 1957; Laurell *et al.*, 1957; Waldenström, 1958; Owen *et al.*, 1959). To be included in this group are some cases of "essential" cryoglobulinaemia (Hutchinson and Howell, 1953; Mackay, Eriksen, Motulsky, and Volwiler, 1956; Volpé, Bruce-Robertson, Fletcher, and Charles, 1956; Barnett, Curtain, and Hayes, 1956; Powers, 1958).

Apart from the above, a number of instances of minor electrophoretic anomalies of the same type have been described. These reports have concerned patients with nephritis (Stern, Mais, and Boggs, 1957), primary amyloidosis (Block, Rukavina, and Curtis, 1956), rheumatic disease (Wallis, 1950; McQueen, O'Shea, and Summerfield, 1954; Salt, 1956), tuberculosis (Matsui and Tamura, 1955), and viral hepatitis (Wajchenberg and Hoxter, 1955). However, others (for references see Owen, 1958a) who have studied the electrophoretic pattern in those conditions have failed to note such anomalies.

Very few systematic studies have been made to date on the anomalous components occurring in most of the conditions listed here under miscellaneous. Accordingly, the remainder of this review is restricted to consideration of the anomalous components associated with myelomatosis, malignant lymphoma, haemolytic disease and, from the miscellaneous group, essential cryoglobulinaemia.

Anomalous Serum Proteins

On electrophoresis, anomalous serum proteins behave as homogeneous proteins appearing on the electrophoretic pattern as narrow "spikes." The electrophoretic mobility of myeloma or lymphoma proteins varies from patient to patient; values obtained for a series of myeloma proteins showed a bimodal distribution, ranging from that of an α_2 globulin to that of a slow γ globulin (Putnam and Udin, 1953). Anomalous components associated with acquired haemolytic disease show less variation in electrophoretic mobility (Fudenberg and Kunkel, 1957); the majority migrate as fast γ globulins. In essential cryoglobulinaemia the anomalous component has been found to migrate as either a fast or a slow γ globulin.

In contrast, γ globulin, which resembles anomalous serum components in many other respects, is electrophoretically heterogeneous. It contains a large number of individual proteins collectively with a range of electrophoretic mobilities and appears on the electrophoretic pattern as a broad hump. It is of interest, however, that the electrophoretic distribution of protein in normal serum precipitating with γ globulin antiserum is much wider than that

*It should be noted that in some of these cases the possibility of coincidental neoplasia, e.g., of the reticulo-endothelial system, was not rigorously excluded.

indicated by the hump on the electrophoretic pattern, approaching that shown collectively by anomalous serum components (Slater, 1955).

In occasional cases, two or more electrophoretically distinct anomalous components were present in the serum (Knedel, 1955; Neely and Neill, 1956; Owen, Rider, and Stewart, 1956; Petermann, Hamilton, and Korngold, 1956; Tsevrenis and Samios, 1958). Similar evidence has been obtained on electrophoresis of myeloma proteins in starch gel (Owen, Got, and Silberman, 1958).

On examination with the ultracentrifuge, some anomalous components behave as if homogeneous, whilst others show heterogeneity even though they appear homogeneous on electrophoresis. Such heterogeneity, however, usually amounts to the presence of a main component with much smaller amounts of other components. In myelomatosis, the main or only component usually has a molecular weight (as indicated by the sedimentation constant*) approximately that of the bulk of normal γ globulin (sedimentation constant $S^{20}_{w}=6$–7 S). In contrast, the anomalous serum components in patients with lymphoma are commonly macroglobulins with higher molecular weights ($S^{20}_{w}=11$–28 S). However, these findings are not invariable. Thus, anomalous serum components with high sedimentation constants have been noted in patients with myelomatosis (Putnam and Udin, 1953; Smith, Brown, McFadden, Buettner-Janusch, and Jager, 1955; Motulsky *et al.*, 1958; Wanner and Siebenmann, 1957; Curtain, 1959a) whilst components with low sedimentation constants have been found in patients in whom the histology was predominantly lymphoid in nature (Owen *et al.*, 1959).

In idiopathic cold-agglutinin haemolytic disease the anomalous components are mostly macroglobulins (Christenson, Dacie, Croucher, and Charlwood, 1957; Fudenberg and Kunkel, 1957). In one such case, however, the amount of macroglobulin in the serum was within normal limits (Owen *et al.*, 1959), i.e., less than 5% of the total protein (Svedberg and Pedersen, 1940). It may be noted that some of the macroglobulin normally in serum behaves as an α globulin while the remainder behaves as a γ globulin (Wallenius, Trautman, Kunkel, and Franklin, 1957; Eriksen, 1958).

Deutsch and Morton (1957) have shown that certain anomalous macroglobulin components can be reversibly broken down under the action of thiol reagents to smaller units (sedimentation constant $S^{20}_{w}=6$–7 S). Similar results have been obtained with cold-agglutinin macroglobulins (Fudenberg and Kunkel, 1957). This suggests that the molecular size of an anomalous protein expresses the tendency of component units to aggregate and apparent heterogeneity on ultracentrifugation represents variable aggregation.

In amino-acid composition, myeloma and lymphoma proteins, though showing individual differences, closely resemble normal γ globulin (Putnam, 1957, 1958). In only one instance has an amino-acid (hydroxy-proline) not detectable in γ globulin been reported in an anomalous protein (Mandema *et al.*, 1955). Anomalous serum proteins likewise resemble normal γ globulin in their N-terminal groups which are usually aspartyl or glutamyl or both. However, some myeloma proteins have been found to have either a leucyl or a phenylalanyl N-terminal grouping (Putnam, 1957, 1958) neither of which has been detected in normal γ globulin (Putnam, 1953; McFadden and Smith, 1953).

The use of carbohydrate stains after paper electrophoresis of serum has indicated that anomalous serum proteins contain carbohydrate (Rice, 1954; Sonnet *et al.*, 1955). This has been confirmed by analysis of anomalous components isolated from serum, which have been found to contain hexose, hexosamine, and sialic acid (Lohss and Hillmann, 1953; Smith *et al.*, 1955; Müller-Eberhard and Kunkel, 1956). The carbohydrate content of components of low molecular weight ($S^{20}_{w}=6$–7 S) is lower than that of components of high molecular weight ($S^{20}_{w}=11$–28 S); in both groups, the components with the mobilities in the β globulin range contain more carbohydrate than those with mobilities in the γ globulin range (Müller-Eberhard and Kunkel, 1956; Laurell *et al.*, 1957).

Normal γ globulin likewise contains carbohydrate. The main component ($S^{20}_{w}=7$ S) contains the same types of carbohydrate, in approximately the same amounts, as low molecular weight anomalous components with the mobilities in the γ globulin range. Low molecular weight anomalous components with the mobilities in the β globulin range contain more carbohydrate. The significance of this, however, is hard to assess since the appropriate fraction of normal proteins with which these faster components should be compared is uncertain (Müller-Eberhard and Kunkel, 1956). The heavy component of normal γ globulin ($S^{20}_{w}=19$ S) contains more carbohydrate than the main component and in this respect resembles anomalous macroglobulins.

*The sedimentation constant of a protein expresses the rate at which its molecules travel in solution under centrifugal force. It forms an approximate measure of the molecular size of a protein.

Sometimes the anomalous components in myelomatosis (Flemberg, 1948 ; Barr, Reader, and Wheeler, 1950 ; Putnam and Udin, 1953 ; Mackay *et al.*, 1956), lymphoma (Waldenström, 1944 ; Lucey *et al.*, 1950 ; Abrams, Cohen, and Meyer, 1949 ; Mackay *et al.*, 1957), or idiopathic haemolytic disease (Christenson *et al.*, 1957) are cryoglobulins, that is, they precipitate spontaneously from serum at temperatures of 0 to 10° C. or higher. Likewise, as has been noted above, in some cases of essential cryoglobulinaemia, the cryoglobulin constitutes a discrete electrophoretic component.

The variable occurrence of small amounts of cold-insoluble protein in pooled normal plasma has been recorded by Morrison, Edsall, and Miller (1948). Cryoglobulins, usually in small amounts, i.e., less than 100 mg./100 ml., have also been found in serum in a variety of diseases, including leukaemia, rheumatoid arthritis, bacterial endocarditis, nephrosis, and kala azar (Holmberg and Grönwall, 1942 ; Wertheimer and Stein, 1944 ; Lerner, Barnum, and Watson, 1947 ; Dreyfuss and Librach, 1952). In some of these cases the isolated electrophoretic cryoglobulin was found to be electrophoretically homogeneous. In most, however, the amount was too small for the cryoglobulin to have appeared as a discrete electrophoretic component in the pattern obtained with whole serum.

Anomalous serum components in myelomatosis (Wuhrmann, Wunderly, and Hässig, 1950 ; Kunkel, Slater, and Good, 1951 ; Deutsch, Morton, and Kratochvil, 1956 ; Korngold and Lipari, 1956a) and in malignant lymphoma (Regniers *et al.*, 1956 ; Korngold and van Leeuwen, 1957) have an antigenic structure related to that of γ globulin, i.e., they have antigenic determinants* which are present also in γ globulin. However, they do not have all the antigenic determinants in γ globulin. Some lack more than others. There is also evidence that anomalous serum components contain patient-specific antigenic determinants (Habich, 1953 ; Slater, Ward, and Kunkel, 1955 ; Kanzow, Scholtan, and Müting, 1955 ; Korngold and van Leeuwen, 1957). Two distinct anomalous serum components with different antigenic structures have been observed in the same serum (Curtain, 1959a).

The question whether anomalous serum components contain antigenic determinants not present in normal γ globulin or in any other serum protein is dealt with later.

*An antigenic determinant is that part of a protein molecule which immunologically stimulates the production of a single antibody. One protein may have several antigenic determinants. Two proteins may have common antigenic determinants.

Anomalous Urinary Components (Bence Jones Proteins)

Unlike any of the serum proteins so far characterized, Bence Jones proteins usually precipitate on heating at temperatures below 60° C. and redissolve, at least partially, on heating to 100° C. Though presumably present in serum, they are most readily detected in urine, often being present there in large amounts.

With boundary electrophoresis or zone electrophoresis on paper, Bence Jones protein in urine usually appears as a single peak or zone. The electrophoretic mobility, however, varies from patient to patient. In some cases traces of other proteins are present as a result of renal damage (Owen, 1957). In occasional cases, the urinary electrophoretic pattern reveals the presence of two or more electrophoretic components (Putnam and Stelos, 1953 ; Berson and Yalow, 1957 ; Owen and Rider, 1958).

In some instances, one of the components appears to be an anomalous serum component which has escaped from the circulation (Owen, 1957). In others the Bence Jones protein appears to comprise more than one electrophoretic component. Electrophoresis in starch-gel has similarly shown electrophoretic heterogeneity in some Bence Jones proteins (Engle, Woods, and Pert, 1957 ; Flynn and Stow, 1958).

Bence Jones proteins have usually a smaller molecular size than do most serum proteins which presumably allows their ready escape from the circulation into the urine. The sedimentation constant varies from case to case though most lie in the range $S^{20}_{w} = 2.5$–4.5 S (Rundles, Cooper, and Willett, 1951 ; Putnam and Stelos, 1953). They show marked individual differences in amino-acid composition (Putnam, 1957). Thus methionine has been reported to be lacking in some Bence Jones proteins (Dent and Rose, 1949 ; Polonovski, Delbarre, and Lévy, 1954) though present in others (Ågren, 1952 ; Deutsch, 1955).

Like anomalous serum components, Bence Jones proteins are antigenically related to normal γ globulin (Korngold and Lipari, 1956b ; Deutsch, Kratochvil, and Reif, 1955 ; Burtin, Hartmann, Fauvert, and Grabar, 1956). However, they too lack some of the antigenic determinants of normal γ globulin.

As might be expected from these findings, Bence Jones proteins and anomalous serum components contain common antigenic determinants. Bence Jones proteins with different antigenic structures have been found in the same urine (Hektoen and Welker, 1940).

Formation and Fate of Anomalous Components

The frequent occurrence of anomalous serum and urinary protein components in myelomatosis and malignant lymphoma has led to the hypothesis, first put forward in respect of myelomatosis (Magnus-Levy, 1933), that anomalous components are formed by myeloma or lymphoma cells. More direct evidence of the presence of anomalous components in myeloma or lymphoma tumour tissues has been obtained by several workers (Martin, 1947; Abrams *et al.*, 1949; Lane, 1952; Miller, Brown, Miller, and Eitelman, 1952; Buffa and Rappaport, 1957). Protein antigenically similar to (and probably identical with) the anomalous serum component has been demonstrated within myeloma cells by means of fluorescent antibody stains (Wollensak and Seybold, 1956; Vazquez, 1958; Curtain, 1959a). Further, isotope incorporation studies have shown that myeloma cells are able to synthesize the anomalous components (Sonnet, 1957; Meyer, 1957). Supporting evidence comes from the discovery in mice of a transmissible plasmacytoma which is associated with an anomalous serum component (Potter, Fahey, and Pilgrim, 1957). Whilst these findings shed light on the pathogenesis of anomalous components in patients with neoplastic conditions, they do not, of course, indicate the source of anomalous proteins in other conditions.

Labelling of anomalous serum proteins in myelomatosis with isotopes has shown that these components have a dynamic existence with half-lives similar to those of other serum proteins (Hardy and Putnam, 1955; Putnam, Meyer, and Miyake, 1956; Osserman, Graff, Marshall, Lawlor, and Graff, 1957; Berson and Yalow, 1957). Their exact fate, however, is unknown. Serial studies indicate that the concentration of an anomalous component in serum is frequently more or less constant over long periods in spite of the progression of the disease (Owen, Rider, and Newall, 1957). Repeated venesections have likewise failed to influence the plasma level significantly (Barr *et al.*, 1950; Soulier, 1950). This suggests that the production and/or destruction mechanism is regulated by the concentration in the plasma. Individual differences in the amount of the anomalous component in plasma imply a different "setting" of the regulating mechanism in each case.

Reiner and Stern (1953) have noted apparent changes in the electrophoretic mobility of myeloma proteins in serum during the course of the disease. However, these were slight and may possibly have been due to changes in the composition of the sample analysed rather than to change in the true mobility of the myeloma protein.

It has been suggested that Bence Jones proteins may represent breakdown fragments of normal or anomalous serum components (Rundles *et al.*, 1951; Osserman and Lawlor, 1955). However, isotopic studies (Hardy and Putnam, 1955; Putnam *et al.*, 1956) indicate that this is unlikely. Moreover, in individual cases, Bence Jones protein and anomalous serum protein are present or absent independently. Data obtained from isotopic studies are, however, consistent with the hypothesis that Bence Jones proteins are precursors of normal or anomalous serum components (Putnam, 1957).

Are the Anomalous Components Normally Produced in Small Amounts?

An answer to this question has been sought by two main routes.

(1) The Chemical Approach.—The aim with this approach has been to determine whether or not the anomalous component has some characteristic, e.g., amino acid, end-grouping, molecular size, not present in any of the normal components of serum. It has been claimed that hydroxyproline was present in an anomalous component, though absent from normal serum proteins (Mandema *et al.*, 1955). Clearly, however, failure to detect a protein with a particular feature in normal serum is of little significance since it is always possible that a more sensitive technique will succeed in this (Martin and Close, 1957).

Most comparative studies have failed to reveal any feature peculiar to anomalous components. Moreover, there has been a tendency in such studies to compare anomalous components with γ globulin or fractions thereof, on the grounds that they resemble γ globulin in other respects, rather than with the proteins of whole serum or, better, plasma.

(2) The Immunological Approach.—The aim with this approach has been to determine whether or not the anomalous component has some immunological characteristic not present in the normal components of plasma. Such studies* fall into two groups: (1) An antiserum is prepared against the purified anomalous protein; this is allowed to react with whole human plasma and the antiserum is then tested for activity against the antigen. If none remains it is concluded that

*Since antigenic specificity presumably stems from molecular configurations, these studies constitute, strictly, part of a "chemical approach." However, they are more conveniently considered separately.

the anomalous component is present in normal plasma.

So far those who have investigated this particular aspect have failed to reach agreement. Some have concluded that myeloma proteins (Wuhrmann *et al.*, 1950; Lohss and Hillmann, 1953; Lohss, Weiler, and Hillmann, 1953; Korngold and Lipari, 1956a) and lymphoma proteins (Boerma and Mandema, 1957; Korngold and van Leeuwen, 1957; Scheidegger, Weber, and Hässig, 1958) contain antigenic determinants absent from normal serums whilst others (Deutsch *et al.*, 1956 Smith *et al.*, 1955; Morton and Deutsch, 1958) have concluded to the contrary. Patient-specific antigenic reactions of anomalous components suggest the view that anomalous components are abnormal. It is possible, however, that there are person-specific components in normal plasma.

However, even if all the antibody activity can be absorbed with normal plasma there remains the possibility that the proteins in plasma may contain all the antigenic determinants present in the anomalous component though not necessarily all in the same molecular species. On this view, the anomalous component would be, antigenically, a hybrid of two or more normal proteins (Curtain, 1959b).

(2) Immunological tolerance to the proteins of normal plasma is produced in a rabbit by neonatal injection. Subsequently the animal is challenged with the anomalous component and the animal's serum examined for antibody to the anomalous component (Curtain, 1959b).

Results of such experiments have suggested (Curtain, 1959b) that all the antigenic determinants of anomalous components are normally present in plasma. However, the possibility that the anomalous components are hybrids, and thus abnormal, is not excluded.

Pathogenesis of Anomalous Components

The mechanisms which have been suggested to account for the production of anomalous components can be set out as follows:

(1) They are formed by proliferating (malignant) cells which are of a type normally producing the anomalous component but are normally present in such small numbers that the anomalous component remains undetectable.

(2) They are formed by proliferating (malignant) cells, progeny of a type which does not normally produce anomalous components, but which start to produce these components as a result of some pathological change.

(3) They are formed by cells which are normally present and which either form normally only insignificant amounts of anomalous components or form them only as a result of some stimulus.

The first possibility amounts to neoplasia of a cell active in the synthesis of protein. Evidence indicating that myeloma or lymphoma cells form anomalous components has already been put forward. The condition would thus be analogous to various "biochemically active" tumours such as the carcinoid tumour, the islet-cell adenoma, or the pituitary adenoma. The variable chemical nature of the anomalous components, however, would mean that there are normally present in the body a great number of cells (presumably of the reticulo-endothelial tissue) each forming a different protein which only becomes detectable when the particular line of cells producing it proliferates extensively. This mechanism is analogous to the clonal selection mechanism developed by Burnet (1957) to account for the production of antibodies. It should be noted, however, that individual antibodies in serum are widely distributed in the electrophoretic spectrum (Loveless and Cann, 1953; Laurell, 1955; Skom and Talmage, 1958), unlike anomalous components which are mostly electrophoretically homogeneous.

Accordingly, each anomalous protein could be a component of γ globulin which is recognized to comprise a large number of molecular species (Saifer and Corey, 1956). This would account for the physico-chemical affinities of γ globulin and anomalous serum components. Moreover, there is evidence (Bing, 1940; Fagraeus, 1948; Coons, Leduc, and Connolly, 1955; Good, 1955; Ortega and Mellors, 1957) that γ globulin is synthesized largely by plasma and lymphoid cells which are, at least morphologically, related to myeloma and lymphoma cells respectively. The selective proliferation of a single cell line required by this hypothesis and the consequent production of detectable amounts of a single protein is to be contrasted with the presumably non-selective proliferation of reticulo-endothelial cells, e.g., plasma cells, occurring in other conditions such as rheumatoid arthritis (Hayhoe and Smith, 1951), infection (Fadem and McBirnie, 1950), or neoplasia (Coste, Bourgeois, Delbarre, and Blatrix, 1948; Clark and Muirhead, 1954). In these conditions there is usually no anomalous serum component though often a generalized hyperglobulinaemia.

The second possibility would involve somatic mutation and offers an alternative explanation for the apparently unlimited variation in the chemical and immunological nature of individual anomalous

components. In considering the possibility that neoplasia represents the outcome of somatic mutation, Armitage and Doll (1954) have concluded that a series of changes must occur before the cell becomes frankly malignant. It is possible that occasionally one such change might cause the cell to produce the anomalous component (Mackay, 1956). This would explain the variable occurrence of anomalous components in patients suffering from the same disease, e.g., myelomatosis.

If this is correct, it would appear that this mutation usually occurs before that inducing frank malignancy, since the first appearance of anomalous serum components after the disease has become established has only rarely been recorded (Owen *et al.*, 1957 ; see, however, Rundles *et al.*, 1951 ; Martin and Close, 1957). On the other hand, a change inducing the formation of an anomalous component could only become evident if sufficient cells of that particular line were present. This would occur when cell division became malignant. Possibly more benign cell division would result in the production of sufficient cells to form detectable amounts of anomalous components without producing anatomical evidence of neoplasia (cf. Case 2, Martin and Close, 1957). This would explain apparently idiopathic cases, e.g., "essential" cryoglobulinaemia.

The possibility that anomalous components were the manifestation of virus infection was considered by Waldenström (1944) and also by Dent and Rose (1949), who pointed out that certain Bence Jones proteins are similar chemically to the protein of tobacco mosaic virus in that they usually lack methionine. However, some Bence Jones proteins contain methionine. Moreover, evidence that myelomatosis or lymphoma is an infective condition has yet to be obtained.

Curtain (1959a), studying a case of myelomatosis in which the serum contained two immunologically distinct myeloma proteins, obtained evidence that they were not formed in the same cell. If this is true of all cases in which two (or more) distinct components are present, it would be necessary according to either the first or the second possibility to postulate selective proliferation of two cell lines (or more), each producing a different protein. This postulate would also be necessary to account for the production of Bence Jones proteins, when these accompany anomalous serum components, unless both proteins are formed in the same cell, a possibility which does not appear to have been investigated.

The mutation theory would appear to offer the most ready solution to this difficulty, on the grounds that the cells from which the two mutated cell lines arose had already undergone a mutation (or mutations) which made all progeny regardless of subsequent mutations prone to become malignant. However, it should be remembered that apparent molecular differences in anomalous components may result from physical phenomena such as molecular aggregation. Observations in this direction have already been reported (Wiedermann, 1958).

Whilst either of the first two possibilities provides an explanation for the association of anomalous components with neoplasia of the reticulo-endothelial system or elsewhere, the third possibility seems necessary to account for cases in which neoplasia are absent. The nature of the stimulus which would cause cells to increase the production of a protein to a detectable level or to produce a protein *de novo* is conjectural. The problem is similar to that posed by the production of antibodies.

In cases in which idiopathic cold-agglutinin haemolytic disease is associated with anomalous serum components the latter have been shown to have anti-erythrocytic properties. It seems likely, however, that their anti-erythrocytic activity is fortuitous and that the condition is not primarily one of auto-immunization. The concentration of anomalous components in serum is often much higher (g./100 ml.) than that usually assigned to antibodies. Further, the agglutinating properties of such proteins are greatest at non-physiological temperatures and they show little specificity towards the source of the erythrocytes whether from different individuals or different species (Dacie, 1954). This is in contrast to the behaviour of warm agglutinins (Dacie and Cutbush, 1954). As has been pointed out (Owen *et al.*, 1959), in Hashimoto's disease, which is much more certainly an auto-immune phenomenon, the serum contains auto-immune antibodies (Witebsky, Rose, and Shulman, 1958 ; Roitt, Doniach, Campbell, and Hudson, 1956), yet the serum electrophoretic pattern does not contain anomalous components, though the γ globulin peak is often increased (Cooke and Luxton, 1955 ; Doniach and Hudson, 1957). The agglutinating activity of certain anomalous components is possibly analogous to the action of certain plant proteins in agglutinating erythrocytes (Landsteiner, 1936), though some of the latter proteins, unlike anomalous serum components, show specificity towards erythrocytes of different ABO blood groups (Renkonen, 1948).

It is of interest that the anti-erythrocytic activity of certain anomalous (macroglobulin) components disappears on dissociation of the

molecule into smaller units (Fudenberg and Kunkel, 1957). However, as these authors point out, the anti-erythrocytic activity is not simply due to the size of the molecule since most anomalous macroglobulin components do not have this property.

Lastly, the possibility that anomalous components, in some instances, constitute a congenital anomaly deserves consideration. Hereditary differences in the protein composition of serum have been revealed by starch-gel electrophoresis (Smithies, 1955, 1958). This could explain the presence of such components in the serum of apparently normal persons or of persons suffering from a disease not usually associated with anomalous components.

Pathological Effects

In patients with anomalous components, certain effects can be attributed to the production of anomalous components and to their presence in serum rather than to any primary disease process which may be present. These effects fall into two groups, those due to the consumption of materials in the synthesis of these components and those which are a direct consequence of the presence of the components in plasma.

The continued synthesis of anomalous components must involve utilization of amino-acids in competition with other tissues. Further, the urinary excretion of such components, e.g., Bence Jones proteins, involves loss of nitrogen from the body. Daily excretion rates of Bence Jones protein as high as 30 g. have been recorded (Hopkins and Savory, 1911 ; Dent and Rose, 1949).

It should be noted, however, that whilst cachexia is common in the conditions in which anomalous components occur, e.g., myelomatosis, lymphoma, it is also prominent in other neoplastic conditions. Further comparison of mean survival times of patients with and without Bence Jones proteinuria have failed to reveal that this loss of nitrogen has a significant effect on survival time (Owen, 1958b). Similarly, no difference has been found in the mean survival times of patients with severe hyperglobulinaemia and those with only moderate hyperglobulinaemia (Owen, 1958).

A reduced amount of normal γ globulin has frequently been noted in patients with anomalous serum components (Adams, Alling, and Lawrence, 1949 ; Rundles *et al.*, 1951 ; Snapper, Turner, and Moscovitz, 1953 ; Waldenström, 1954 ; Zinneman and Hall, 1954 ; Lawson, Stuart, Paull, Phillips, and Phillips, 1955 ; Porges, 1956 ; Firkin and Blackburn, 1958 ; Owen *et al.*, 1959), and this finding is probably more common than such reports indicate, since the anomalous component frequently migrates as a γ globulin, thus obscuring any diminution in normal γ globulin. Hypogammaglobulinaemia in such patients may reflect a competition for raw materials between cells forming normal γ globulin and those forming an anomalous component, especially in view of the chemical similarities of anomalous components and normal γ globulin. However, other factors are almost certainly involved. Thus hypogammaglobulinaemia has been observed in patients with myelomatosis, lymphoma, or leukaemia (Arends, Coonrad, and Rundles, 1954 ; Jim and Reinhard, 1956 ; Wall, 1956 ; Citron, 1957) in whom anomalous serum or urinary components were not present. This might suggest that competition is for space rather than for raw materials. On the other hand, many patients with lymphoma or leukaemia have normal or even increased serum γ globulin levels (Rundles *et al.*, 1951 ; Jim and Reinhard, 1956 ; Owen *et al.*, 1956).

The occurrence of a haemorrhagic tendency, manifested as bruising, bleeding from mucosal surfaces, or haemorrhagic retinitis, in association with anomalous serum components is well documented (Waldenström, 1944 ; Craddock, Adams, and Figueroa, 1953 ; Frick, 1955 ; Long, Riopelle, Francoeur, Pare, Poirier, Georgesco, and Colpron, 1955 ; Jim and Steinkamp, 1956). This has been attributed either to a defective coagulation mechanism or to an effect of the anomalous serum components on capillary permeability (Waldenström, 1952). Direct interaction of anomalous components with coagulation factors has been demonstrated in some cases (Henstell and Feinstein, 1957 ; Firkin, 1958). Thrombocytopenia, however, is often present, and is presumably a contributory factor (Frick, 1955 ; Jim and Steinkamp, 1956 ; Mackay, 1956).

Cardiac failure in such patients has been attributed to a high blood viscosity resulting from the presence of anomalous components in plasma (Barr *et al.*, 1950 ; McFarlane, Dovey, Slack, and Papastamatis, 1952). A thrombotic tendency which, in some cases, may paradoxically accompany a haemorrhagic tendency, has also been attributed to a high blood viscosity (Frick, 1955) and is partly responsible for the retinitis present in some cases. Thrombosis of intracranial vessels is presumably also responsible in part for neurological and psychological symptoms, though there may be infiltration with neoplastic cells (Mackay, 1956). Where the anomalous component

is a cryoglobulin, blood viscosity may be greatly increased in exposed areas as a result of intravascular gellification or precipitation of the cryoglobulin. Local circulatory failure consequent on this gives rise to Raynaud's phenomenon and peripheral gangrene.

An increased erythrocyte sedimentation rate is usual, though not invariable (Owen and Rider, 1957), in patients with anomalous serum components. Presumably the high sedimentation rate is due to interaction between anomalous components and erythrocytes.

Renal failure in myelomatosis is attributable to the deposition of protein within the kidney (Snapper *et al.*, 1953; Allen, 1951). Rarely protein as para-amyloid is deposited in the glomeruli. More commonly protein accumulates within the tubules. There is, however, little correlation between the degree of hyperglobulinaemia or proteinuria and the extent of renal impairment (Allen, 1951). Amino-aciduria occurring in myelomatosis (Engle, 1956; Sirota and Hamerman, 1954) is perhaps due to direct competition between the tubular absorption of amino-acids and of anomalous components, especially Bence Jones proteins which presumably escape from the plasma more readily than anomalous serum components.

False positive Wassermann reactions have been noted in certain sera containing anomalous components (Long *et al.*, 1955; Martin and Close, 1957; Zlotnick, 1958) whilst a related reaction (AICF) has been observed in other cases (Mackay and Gajdusek, 1958; Mackay, 1959). Some sera with anomalous components have proved anticomplementary (Snapper *et al.*, 1953; Volpé *et al.*, 1956). It is not known, however, whether these reactions with complement are attributable directly to the presence of the anomalous component or, indirectly, to the underlying disease process.

Conclusions

Anomalous serum and urinary components occur most commonly in myelomatosis and lymphoma. They also occur occasionally in a number of other conditions. In a few cases, no histological evidence of disease can be obtained which suggests that such proteins are manifestations of a primary lesion at a biochemical level. Certain morphological changes are probably secondary to the presence of the anomalous components.

In some of the conditions in which they have been found, incomplete data concerning occurrence of anomalous components make it yet impossible to assess their significance. In cases associated with malignant cell division, however, the production of anomalous components can best be attributed either to the selective proliferation of normal cells, i.e., cells which normally form the anomalous component in small amounts, or to the selective proliferation of abnormal cells, i.e., cells which have undergone a somatic mutation conferring on them the ability to produce the anomalous component.

The choice between these two possibilities depends largely on whether anomalous components are qualitatively normal or abnormal. As yet evidence does not allow a clear decision on this issue. Indeed, whilst it may be possible to prove that anomalous components are normally present in small amounts it is virtually impossible to prove that they are not.

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CAERULOPLASMIN AS DEMONSTRATED BY STARCH GEL ELECTROPHORESIS

BY

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Starch gel electrophoresis has demonstrated that graded concentrations of sodium sulphate do not precipitate the serum globulins in any reasonable degree of purity.

The use of this form of electrophoresis has shown that caeruloplasmin is associated with a globulin in the $F\alpha_2$ position.

This technique can be employed as a screening test for patients suspected of suffering from hepatolenticular degeneration.

Thompson and Watson (1949), utilizing graded concentrations of sodium sulphate for precipitating the various globulins of blood serum, demonstrated that copper appeared to be linked with each of the three main globulins. These observations were confirmed by Cumings, Goodwin, and Earl (1955) in normal subjects, and they also reported on the findings in patients with hepatolenticular degeneration using the same procedure. The oxidase activity of each globulin fraction in normal subjects was also estimated, using paraphenylenediamine as the substrate, and most activity was detected in the β fraction.

These findings are at variance with a commonly-accepted view that caeruloplasmin, the copper-containing globulin possessing oxidase activity, is an α_2 globulin complex.

The introduction of starch gel electrophoresis as developed by Smithies (1955) and the possibility of staining such gels for caeruloplasmin suggested a new approach to the problem, and the results of some investigations on these lines are recorded in this paper.

Methods

Starch electrophoresis was carried out by the method of Smithies (1955) using a block measuring $22.5 \times 3.8 \times 0.6$ cm. and run overnight at a voltage of 120 with a current of 0.44 mA/cm. width. After cutting the block as recommended it was stained for protein in the manner described by Smithies. For the staining of caeruloplasmin the technique of Uriel (1958) was followed.

Globulin fractions were prepared as in the previous paper, and they and serum were absorbed on to small pieces of Whatman 3MM paper, and these were inserted into the starch blocks. In some experiments paper electrophoresis of serum in duplicate was carried out. One of the two strips was stained for protein and appropriately sized pieces of paper from the unstained strip were cut out to correspond with α, β, or γ globulin and inserted into starch blocks.

Results

Electrophoresis of normal serum in starch yielded results similar to those obtained by Smithies, and his terminology is used throughout.

It was found that the globulin fractions, as prepared by sodium sulphate precipitation, when run in starch were not as pure as they appeared to be on paper electrophoresis. Four such experiments were performed and all gave similar results, one of which is illustrated in Fig. 1.

The strip representing γ globulin (15% salt) is seen to show only that globulin by both methods of electrophoresis. The fraction containing γ and β globulin (19% salt), although apparently only containing these globulins on paper, is seen in starch to contain in addition a well-marked $S\alpha_2$ globulin band and a small amount of $F\alpha_2$ globulin.

All the globulins are present in the starch gel and on paper electrophoresis in the fraction precipitated by 26% sodium sulphate; there is, however, more albumin demonstrated in starch than on paper.

All these various starch blocks were also stained for caeruloplasmin, and while no activity, as indicated by the brown colour of the Bandrowski base, was seen in the fraction precipitated by 15% salt, it was present in both the other two fractions. This test is inconclusive

in view of the mixture of globulins shown by the results of the starch electrophoresis.

In order to determine the site of caeruloplasmin relative to the globulins two techniques were employed. First, portions of paper each containing one of the globulins obtained from paper electrophoresis of serum were inserted into starch blocks, run overnight and stained for protein and for caeruloplasmin. Fig. 2 demonstrates one of the five results obtained.

A stained band of caeruloplasmin is present in serum and in the α globulin fraction and appears in the $F\alpha_2$ position in the starch gel. No such stained band was obtained in any experiment from either the β or the γ portions of the paper electrophoretic strips.

The second method employed was to run serum in a starch gel, and, before dividing the gel into two portions, to punch out a series of holes along the starch block. This is necessary, as during the process of protein staining the gel contracts a little, so that it is not possible otherwise to match exactly the two strips stained by both methods. The starch blocks were then stained, and, by noting the positions of the various bands in relation to the number of the holes, it was demonstrated in three cases that the caeruloplasmin band corresponded to the $F\alpha_2$ position.

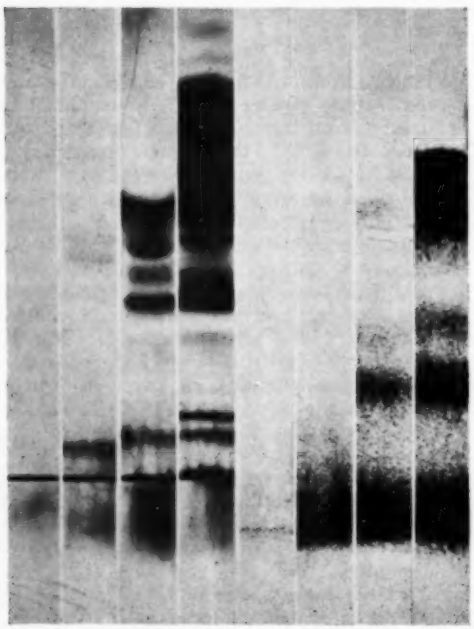

FIG. 1.—Normal serum and globulins obtained by salt precipitation run in starch (left) and on paper (right). The four paper strips are in the same order as the starch gels.

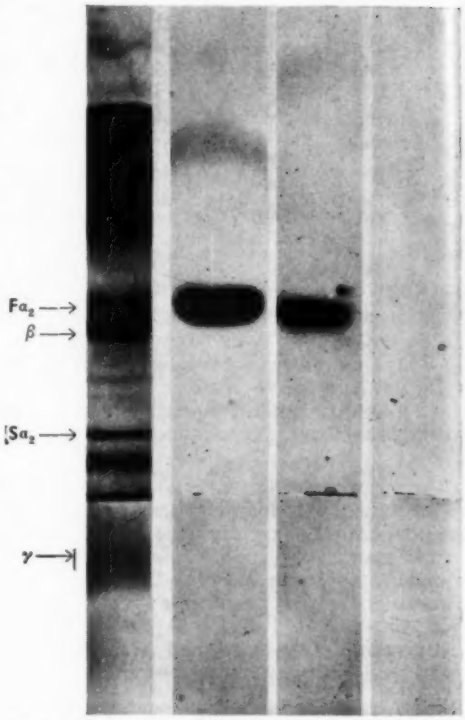

FIG. 2.—Starch gel electrophoresis of normal serum (two on left), of α globulin (second from right), and of β globulin (on right). The three on the right stained for caeruloplasmin and the one on the left for protein.

Discussion

It has been stated by Bearn and Kunkel (1954) as a result of their experiments that the copper in the blood is attached to an α_2 globulin. Holmberg and Laurell (1948, 1951) considered that the compound they isolated from human and pig blood and which they named caeruloplasmin was an α globulin. Thompson and Watson had suggested from their experiments that copper could be bound to each of the three main globulins and we had supported this view by other earlier experiments. This opinion we find no longer tenable in view of the results recorded here, for it would appear to be established that caeruloplasmin is in the $F\alpha_2$ position and that the globulins as prepared by graded sodium sulphate

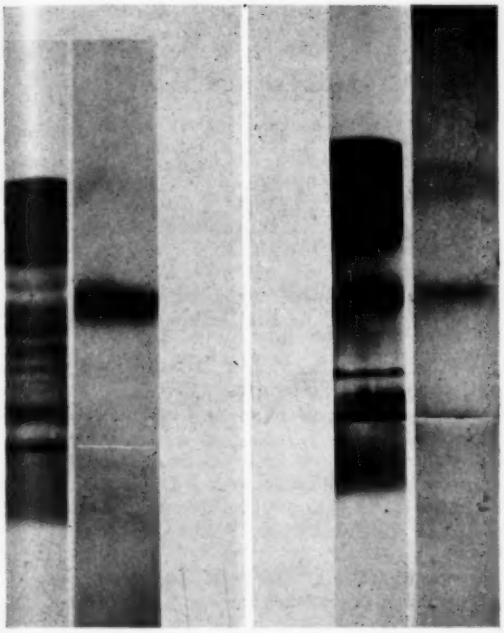

FIG. 3.—Starch gel electrophoresis of serum from a normal control (left) and from a patient with hepatolenticular degeneration (right) stained for protein and caeruloplasmin.

precipitation are not as pure as had appeared on paper electrophoresis.

Uriel has described his findings relating to caeruloplasmin using a specific staining technique, but his preparations have been with agar and not starch.

The method employed by us might well be a useful screening technique in patients suspected of suffering from Wilson's disease. If serum is run in starch blocks and stained for both protein and caeruloplasmin, a comparison can be made between the sera of a normal control and of the patient. Fig. 3 demonstrates one of the cases which we have investigated together with a normal control and shows very clearly the difference between the two sera. It is also seen that both stained areas are in the $F\alpha_2$ position.

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FLOCCULATION TESTS IN HYDATID DISEASE

BY

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A new serological technique of diagnosing human hydatid disease, employing polystyrene latex particles coated with hydatid cyst fluid, and a modified technique using bentonite particles are described.

Comparison of the latex test with the complement-fixation test performed on 102 sera has shown a total agreement of 98% and an agreement of 93.1% in the positive specimens. The bentonite test and the complement-fixation test performed on 126 sera have shown a total agreement of 97.6% and an agreement of 90.1% in the positive specimens.

Both flocculation tests are simpler to perform than the complement-fixation test. They are specific, and sensitivity closely parallels that of the complement-fixation test, with 6.9% and 6.1% of the positive specimens showing higher sensitivity in the latex and bentonite tests respectively. The bentonite test was less sensitive than the complement-fixation test in one case. None of the latex tests showed less sensitivity.

The antibody active in the latex test has been demonstrated in a γ globulin fraction of the antiserum. Some differences in the behaviour of flocculating and complement-fixing properties of sera are discussed.

The complement-fixation test has been employed routinely in the diagnosis of human hydatid disease for many decades (Dew, 1928; Fairley and Kellaway, 1933; Bensted and Atkinson, 1953). Other serological methods simpler than complement fixation have been reported. Thus Fairley (1923) claimed good results with a direct precipitin test. Subsequent authors found this test difficult to read and less sensitive than the complement-fixation test (Bryce, Kellaway, and Williams, 1924; Dew, 1928; Fairley and Kellaway, 1933). Recently an indirect haemagglutination test was described by Garabedian, Matossian, and Djanian (1957).

In the present paper two methods are described using particles, other than red cells, coated with antigen. Results are compared with the complement-fixation test. These techniques are simple, easy to read, and sensitive. The first procedure employs polystyrene latex particles, introduced by Singer and Plotz (1956) for the detection of the rheumatoid arthritis factor. The second test is an adaptation of the bentonite technique devised by Bozicevich, Tobie, Thomas, Hoyem, and Ward (1951) to diagnose trichinosis, and employed by Sadun, Norman, Allain, and King (1957) to test sera of animals inoculated with *Echinococcus multilocularis*. These authors suggested a possible application of the bentonite technique in human hydatid disease.

Methods

Antigen.—Fluid removed aseptically from sheep hydatid cysts is pooled and stored frozen at −20° C. in small aliquots. Immediately before use the fluid is thawed and centrifuged at 3,000 r.p.m. for 15 minutes. The supernatant is employed as antigen in all techniques described.

Potency is retained for at least 12 months. Suitability for both of the flocculation tests and the complement-fixation test is checked initially and at regular intervals using the same stock positive serum, also stored at −20° C.

Latex Hydatid Test (LHT).—The following are the materials and the method.

Stock Latex.—Polystyrene latex suspension, particle size 0.81 micron diameter (Dow Chemical Co., Midland, Michigan).

Dilute Stock Latex.—A small sample of the stock latex is diluted with distilled water until 0.1 ml. in 10 ml. of water gives 5% transmission in a "unicam" spectrophotometer. This gives the dilution factor. The dilute latex is prepared by diluting 1 ml. of the stock latex with the appropriate amount of water (1.5 ml. water has been used in this study). This suspension keeps well at 4° C.

Sensitized Latex.—Dilute stock latex, 0.1 ml., is added to 10 ml. of physiological saline, mixed gently, then 0.5 ml. of antigen is added and mixed gently.

Procedure.—Sera are cleared by centrifuging if necessary. Add 0.5 ml. of fresh serum to 1.5 ml. of physiological saline in the first tube (1:4). Doubling dilutions are prepared in 1 ml. volumes, and 1 ml. sensitized latex is added to each dilution. The tubes are mixed by inversion, incubated at 37° C. for 90 minutes, and then kept at 4° C. overnight. Next day the tubes are centrifuged at 2,300 r.p.m. in an MSE "major" centrifuge for three minutes and read with the naked eye. A known positive serum diluted 1:8 and a saline control are tested simultaneously.

Positive tests have a wide, heavy deposit. On tapping, suspended flocs are easily visible to the naked eye. The test is considered strong when the supernatant is clear, and the resuspended flocs large; weak when the supernatant is slightly hazy and the flocs smaller. Grading flocs from 1 to 4+ did not seem necessary. Negative tests show an opaque appearance, either without deposit, or with a small deposit easily dispersed by tapping. Occasionally tests appear doubtful, if graded on the appearance of the deposit only. They are considered negative if on resuspension no flocs are visible to the naked eye.

Bentonite Hydatid Test (BHT).—The procedures for making up the necessary solutions and the test itself are as follows:

Stock Bentonite.—This is prepared by the method of Bozicevich *et al.* (1951) using Volclay BC micron bentonite, American Colloid Co., Chicago, 10, Ill. The suspension may be kept at 4° C. for at least nine months.

Stock Sensitized Bentonite.—To 10 ml. of the well-shaken stock bentonite 6 ml. antigen is added, mixed, and left overnight at 4° C. Then 2 ml. of a 0.1% aqueous solution of methylene blue is added, the suspension thoroughly shaken, and allowed to stand for one hour at room temperature. This suspension is kept at 4° C.

Working Sensitized Bentonite.—Ten millilitres physiological saline is added to 8 ml. of the stock sensitized bentonite, mixed and centrifuged for five minutes at 2,500 r.p.m. in an MSE "major" centrifuge. The supernatant is discarded, 15 ml. physiological saline added to the deposit, mixed and centrifuged as before. The supernatant is discarded and the deposit resuspended in 4 ml. physiological saline. Finally 0.1 ml. of a 1% solution of Tween 80 is added. The suspension is tested with a known positive, a negative serum, and a saline control. The amount of Tween 80 added may have to be increased if spontaneous flocculation occurs in the saline control. Working sensitized bentonite is kept at 4° C. and retains its potency for at least two months.

Procedure.—Starting with a 1:4 dilution, serial two-fold dilutions of serum in physiological saline, using 0.1 ml. volumes, are made in 6 by 1.5 cm. tubes. The working sensitized bentonite is shaken vigorously, and one drop is added to each dilution using a capillary pipette delivering approximately 40 drops per millilitre. After the tubes are shaken in a Kahn shaker for 10 minutes, the contents of the tubes are transferred to slides, allowed to stand for three to six minutes for full development of flocs, and examined under the low power of the microscope. A 1:8 dilution of a known positive serum and a negative control are tested simultaneously. The test is graded 4+ if all particles are clumped into separate flocs; 3+ if three-quarters; 2+ if half; 1+ if one-quarter and 0 if none are clumped together. Only 3+ and 4+ are regarded as positive tests, while 2+ to 0 are regarded as negatives.

Complement-fixation Test.—The method used is essentially the Richardson-Wyler modification of the Wassermann technique, except that complement, haemolysin, and cells are standardized as reported before from this laboratory (Whillans, 1950). Antigen is used in two strengths, undiluted and diluted 1:2. Reagents are added in 0.25 ml. amounts. A strong positive test gives complete fixation with both strengths of antigen; a weak positive test gives 75% fixation in one strength, and complete fixation in the other; a doubtful test gives 75% fixation with both antigens. Less than 75% fixation is regarded as a negative reaction. All necessary controls are tested.

Materials and Results

One hundred and six sera received for the routine performance of the hydatid complement-fixation test, and 20 sera received on request, were tested initially simultaneously with the bentonite hydatid test, and later with both the latex and bentonite tests. Thirty sera had a positive and one a doubtful positive complement-fixation test. In 26 of these cases hydatid disease has been confirmed surgically, but in the remaining five it was not possible to obtain information.

Comparison of Complement-fixation and Latex Hydatid Tests (102 Sera).—All sera with a positive complement-fixation test in this series (27) and the doubtful positive one had a positive latex test. All negative complement-fixation tests were accompanied by negative latex hydatid tests, with one exception. This specimen, from a case of hydatids surgically confirmed, gave a positive latex test. This gives a total agreement of 98%, and 93.1% in the positive cases.

Comparison of Complement-fixation and Bentonite Hydatid Tests (126 Sera).—A positive complement-fixation test was accompanied by a positive bentonite hydatid test in all 30 sera. The serum with the doubtful complement-fixation test gave a negative bentonite hydatid test reaction. Negative complement-fixation tests were accompanied by a negative bentonite test, with two

exceptions. These two sera, obtained from patients shortly after removal of a liver and lung hydatid cyst respectively, gave a positive bentonite hydatid test. This gives a total agreement of 97.6% and 90.9% in the positive cases (Table I).

TABLE I

COMPARISON OF POSITIVE RESULTS OF COMPLEMENT-FIXATION, LATEX HYDATID, AND BENTONITE HYDATID TESTS

Complement-fixation and Latex Tests in 29 Cases		Complement-fixation and Bentonite Tests in 33 Cases	
Results	No.	Results	No.
Both + ...	27 (93·1%)	Both + ..	30 (90·9%)
CFT ±		CFT ±	
Latex + ..	1 (3·45%)	Bentonite − ..	1 (3·0%)
CFT −		CFT −	
Latex + ..	1 (3·45%)	Bentonite + ..	2 (6·1%)
Disagreement ..	2 (6·9%)	Disagreement ..	3 (9·1%)

Comparison of Latex and Bentonite Hydatid Tests.—Positive and negative tests ran parallel, except in two cases, one test each being positive while the other flocculation test gave a negative result.

Titres.—These ranged from 1 : 10 to 1 : 60 for the complement-fixation test, from 1 : 8 to 1 : 256 for the latex hydatid test, and from 1 : 4 to 1 : 128 for the bentonite hydatid test. There was no definite correlation between titres of the three tests (Table II) or grade of positivity.

TABLE II

TITRES OF SIX SERA IN THREE HYDATID TESTS

Complement-fixation Test	Latex Test	Bentonite Test
1 : 20	1 : 128	1 : 64
1 : 10	1 : 64	1 : 64
1 : 60	1 : 64	1 : 128
1 : 40	1 : 64	1 : 64
1 : 10	1 : 256	1 : 16
0	1 : 16	1 : 8

For routine testing three dilutions, 1 : 4, 1 : 8, and 1 : 16, were found sufficient for both flocculation tests. Positive tests may be titred to the endpoint. In occasional cases zone inhibition occurred at a dilution of 1 : 4 but not at higher dilutions.

Effect of Heat.—Both fresh sera and sera inactivated at 56° C. for 30 minutes as for the complement-fixation test were found suitable for the bentonite hydatid test. Inactivated serum was less suitable for the latex hydatid test. Inactivation reduced the titre considerably. The temperature of the test is also of importance: 56° C., as used for the rheumatoid latex test (Singer and Plotz, 1956), rendered the latex hydatid test negative, or positive at a minimal dilution only. Fresh specimens incubated at 37° C. for the test, followed by 4° C. overnight, gave best results. The omission of overnight standing resulted in a loss of sensitivity (Table III).

TABLE III

EFFECT OF HEAT ON LATEX HYDATID TEST

Test Temperature 90 Minutes	Additional Incubation	Titres of Sera		
		Fresh	Inactivated 30 Minutes	
			56° C.	60° C.
Room ..	Overnight 4° C.	1 : 16	1 : 8	Undiluted
37° C. ..	,,	1 : 32	1 : 8	,,
56° C. ..	,,	0	1 : 4	0
37° C. ..	None	0	0	0

Effect of Varying Amounts of Antigen.—The optimum minimal amount of cyst fluid to be used for one batch of sensitized particles is given above. Smaller amounts produced less satisfactory results. Table IV shows that in some strongly reacting sera reduction of the amount of antigen still gave sufficient sensitivity, while in another specimen it rendered the latex hydatid test negative.

TABLE IV

EFFECT OF VARYING AMOUNTS OF ANTIGEN ON LATEX HYDATID TEST IN THREE SPECIMENS

Latex Coated with Cyst Fluid (ml.)	Titres		
0·5	1 : 64	1 : 256	1 : 64
0·4	1 : 64	1 : 256	1 : 64
0·25	0	1 : 64	1 : 8
0·5 (diluted 1 : 2)	0	1 : 64	0

Discussion

While bentonite particles have been sensitized with helminthic antigens, latex particles have not been coated with such antigens in the past. The present study has demonstrated successful sensitization of latex particles with hydatid antigen, and application of both latex and bentonite particles in simple diagnostic procedures for human hydatid disease.

This study is essentially a comparison of results with complement-fixation and flocculation tests. As the percentage distribution of positive and negative complement-fixation tests in hydatid disease and its relation to the Casoni intradermal tests have been the subject of numerous reports (Dew, 1928; Fairley and Kellaway, 1933; Bensted and Atkinson, 1953), no attempt was made to ascertain how many of the specimens negative with all tests were from patients with hydatid disease and with disease other than

hydatid. In all cases with positive tests diagnosis was ascertained whenever possible. Flocculation tests closely parallel complement-fixation tests. They are simpler, and seem suitable as alternative or screening tests where facilities for complement fixation are not available. While it appears possible that the flocculation tests are slightly more sensitive than the complement-fixation test, no definite statement to that effect seems permissible, until more cases with surgical confirmation and with diverging results are available.

The latex hydatid test has the advantage of a simpler preparation of sensitized particles and reading with the naked eye. The bentonite hydatid test is more rapid, once the stock and sensitized particles have been prepared. No definite superiority of one flocculation test over the other as regards sensitivity has been established.

The grading of 3+ and 4+ as positives only as for the bentonite trichinosis test (Bozicevich *et al.*, 1951) corresponded well with results for the bentonite hydatid test. Grade 2 readings were infrequent, and were accompanied by negative latex and complement-fixation tests. Of four sera with grade 2 readings two were from patients operated on more than 10 years previously. There was no evidence of recurrence. In the remaining two cases no evidence of past or present hydatid disease was found.

In the Bozicevich technique serial dilutions were transferred to slides and placed on a rotating apparatus. In this laboratory shaking of serial dilutions in tubes in a Kahn shaker and transferring the contents to slides for reading has been found equally satisfactory. The number of steps involved are the same.

The flocculation tests appear to be specific. The complement-fixation test has been considered specific, but rare false positive results have been reported, with the suggestion that selected cyst fluid suitable in higher dilutions may eliminate false positives (Bensted and Atkinson, 1958). Pooled fluid used in this study was unsuitable in higher dilutions for the tests described. Although no false positives have been encountered, the suitability of selected potent fluids in higher dilutions for the flocculation tests will be the subject of further study.

The effect of temperature on the latex test is of some interest. Inactivation by heating sera at 56° C. reduced the titre considerably. Preheating to 60° C. rendered the test negative or positive in the undiluted serum only. A peculiar effect was the complete negativity of the test on fresh sera when the test was carried out at 56° C., while giving a low positive titre with a test temperature of 56° C. on previously inactivated sera. Some of these findings are similar to those reported by Bryce *et al.* (1924) on precipitin tests, showing a gradual decrease in the activity of the precipitating antibody of sera when they were preheated, with total loss at 60° C. No such effect was observed on the complement-fixing property.

Little work has been done since on the nature of the hydatid antibodies. Recently Magath (1959) obtained a positive complement-fixation test on a γ globulin fraction of a serum after electrophoretic separation, with no activity in the other fractions. Precipitin tests in double diffusion agar were performed on whole serum with antigenic fractions, but not with fractions of antiserum. In a preliminary experiment in this laboratory, γ globulin fractions were prepared from three positive hydatid and three negative sera, as described in a previous paper (Whillans and Fischman, 1958). The latex test was positive in the fractions of the positive and negative in the control sera, with no activity in any of the supernatants.

As there is evidence of more than one antigen being present in cyst fluid (Senekji, 1941; Magath, 1959), further work is needed to characterize the antibodies involved, and their relation to the hydatid antigens.

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THE INVESTIGATION OF LIPOPROTEINS IN LYMPH AND OTHER BODY FLUIDS BY PAPER ELECTROPHORESIS

BY

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The protein and lipid electrophoresis patterns of lymph and various body fluids have been examined. Chylous fluids produce a characteristic lipid pattern with a dense deposit at the origin and a lipid trail. The value of centrifuging turbid fluids at high speed before electrophoresis is stressed.

During the course of work on the treatment and pathology of lymphatic disease a number of specimens of lymph were collected and examined by paper electrophoresis. The protein and lipid patterns obtained have been compared with those from various other body fluids, and a few additional simple investigations made to assist in the understanding of the lipid patterns.

As far as we are aware the lipid patterns of human lymph and body fluids have not been previously reported, although Courtice and Morris (1955) investigated the proteins and lipids of animals and their results are, in general, similar to ours. The protein contents and electrophoretic patterns of body fluids have been investigated by various workers, e.g., Crockett (1956) and Taylor, Kinmonth, and Dangerfield (1958).

Methods

Collection of Lymph.—Normal lymph was collected by needle aspiration from either the thoracic duct in the neck or one of the other major cervical lymph channels during the course of routine surgical operations.

Abnormal lymph containing chyle was obtained in a similar way during operation upon abnormally large trunks in the pelvis or groin of patients suffering from chylous reflux and lymphoedema. In these patients the retroperitoneal lymphatic trunks are congenitally dilated and incompetent and allow retrograde flow of lacteal fluid. With this abnormality lymphatic vesicles often occur on the skin of the thigh and perineum, and these leak a chylous fluid. Examination of fluid from such a vesicle is referred to below.

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Laboratory Methods.—The specimens of lymph were taken to the laboratory in the syringe in which they were collected and paper electrophoresis started without delay employing the methods previously described (Dangerfield and Smith, 1955), using light green SF and sudan black B for staining protein and lipid respectively.

Towards the end of this investigation a high-speed centrifuge was acquired and a few specimens were centrifuged at speeds up to 12,000 r.p.m.; usually the specimens were layered under water and centrifuged, when the fat particles migrated to the top of the water layer and could be separated. Very small quantities of fat can be detected in this layer, particularly if a transparent centrifuge tube (glass or nylon) is used and the column of liquid examined in a powerful beam of light.

Assessment of Patterns.—The usual five protein bands were easily identified, but with the lipid patterns there is some difficulty in identification. We attempted to discern five lipid bands as follows:

LDO —Lipid deposit at origin (due to chylomicrons)
LTrail—Lipid "trail" extending from origin to β lipoprotein and sometimes beyond (probably due to smaller fat particles)
β-LP —β lipoprotein, a narrow band level with β globulin
α-LP —α lipoprotein level with α_1 globulin
LA —"Lipalbumin" (probably due to "free" (unesterified) fatty acid adsorbed on albumin)

It is very difficult to decide whether staining in the β globulin region should be attributed to β lipoprotein or to the lipid "trail." In normal sera patterns the β lipoprotein is a sharp, well-defined band whereas the trail is a wide band seen most characteristically in lipaemic sera and chylous fluids. This difference does not provide a satisfactory method of distinction, because the sharpness and even the position of the bands vary slightly with unavoidable

TABLE I
SUMMARY OF RESULTS

Fluid	No. of Samples	Approximate Protein Content (%)	Main Protein Characteristics	Main Lipid Characteristics
Lymph				
Cervical ..	4		Similar to serum but weak α_2 globulin	Usually a weak β lipoprotein. Contaminated with blood
Thoracic duct	7		,, ,,	Dense deposit at origin and trail; β lipoprotein difficult to assess
Chylous inguinal	4		,, ,,	As thoracic duct lymph
Clear fluids				
(a) Oedema fluid				
Heart failure	4	½–1	Weak albumin, v. faint β and γ globulin	Nil
Lymph-oedema	12	½–3	Stronger bands than with heart failure fluids; α_2 globulin relatively weak	Nil or trace
(b) Hydrocele fluid	6	4–6	Similar to serum but α_2 globulin low	Usually some lipalbumin and occasionally α lipoprotein. No β lipoprotein
(c) Pleural fluid				
Heart failure	6	1½–3	Very weak serum pattern	Traces of lipid
Carcinoma	7	4–5	Similar to serum	Similar to serum
Other malignant disease	3	2–5	,, ,,	,, ,,
(d) Ascitic fluid				
Heart failure	4	2–4½	Weak serum pattern, α_2 globulin low	Like dilute serum
Cirrhosis ..	9	½–2½	Weak albumin and weak diffuse γ globulin, only trace of α_2 and β globulin	Traces only
Carcinoma	5	4–5	Like serum	Like serum
(e) Bursa fluid	4	—	,, ,,	,, ,,
(f) Urine (nephrotic)	3	—	Varied protein patterns but α_2 globulin very low compared with patient's serum	Nil
(g) Spermatocele fluid	2	½	Albumin and tr. β globulin	Nil
(h) Amniotic fluid	2		,, ,,	,,
(i) Bladder fluid	1	2½	Ill-defined pattern. Mainly albumin	,,
(j) Hernial sac fluid	1	½	Weak albumin and tr. β globulin	,,
(k) Branchial cyst fluid	1	—	Strong albumin and deposit at origin; some β globulin. Ill-defined pattern	Intense lipid deposit at origin, rich in cholesterol
(l) Parotid fluid	1	—	Albumin and deposit at origin. Faint globulin bands	Nil
(m) Vitreous humour	1	—	Faint deposit at origin only	,,
Turbid fluids				
(a) Chylous ascitic fluid	7		Like weak serum	Dense deposit at origin; trail extends to α_2 globulin front. β lipoprotein difficult to assess
(b) Chylous pleural fluid	1		As above	As above
(c) Chylous blister fluid	1		Like serum	,, ,,
(d) Chylous urine	3		Albumin and faint β globulin and deposit at origin	Very dense deposit at origin; trail very short or absent; no β lipoprotein
(e) Ovarian cyst fluid	1		Ill-defined pattern, fairly high protein content	Strong deposit at origin, some lipalbumin

variations in electrophoretic technique, e.g., density of application and age of specimens. High-speed centrifugation helps in distinguishing these lipids, for the trail lipids are of low density and can be partly spun up into an overlying layer of water, whereas the β lipoprotein remains in the lower layer.

The separation of α lipoprotein and "lipalbumin" is also not entirely satisfactory. In spite of these limitations useful information can be gained from the lipid patterns, and their value grows as technique improves and as more patterns accumulate for comparison.

Results

An examination was made of 15 samples of lymph, four from the cervical lymph ducts, seven from the thoracic duct of patients with a normal lymphatic system, and four from the inguinal lymphatics of patients with chylous reflux. These have been compared with 72 clear body fluids and 13 turbid fluids. The clear fluids were mainly oedema, ascitic and pleural fluids, and the turbid ones were mainly chylous ascitic fluid and urine. The findings are summarized in Table I.

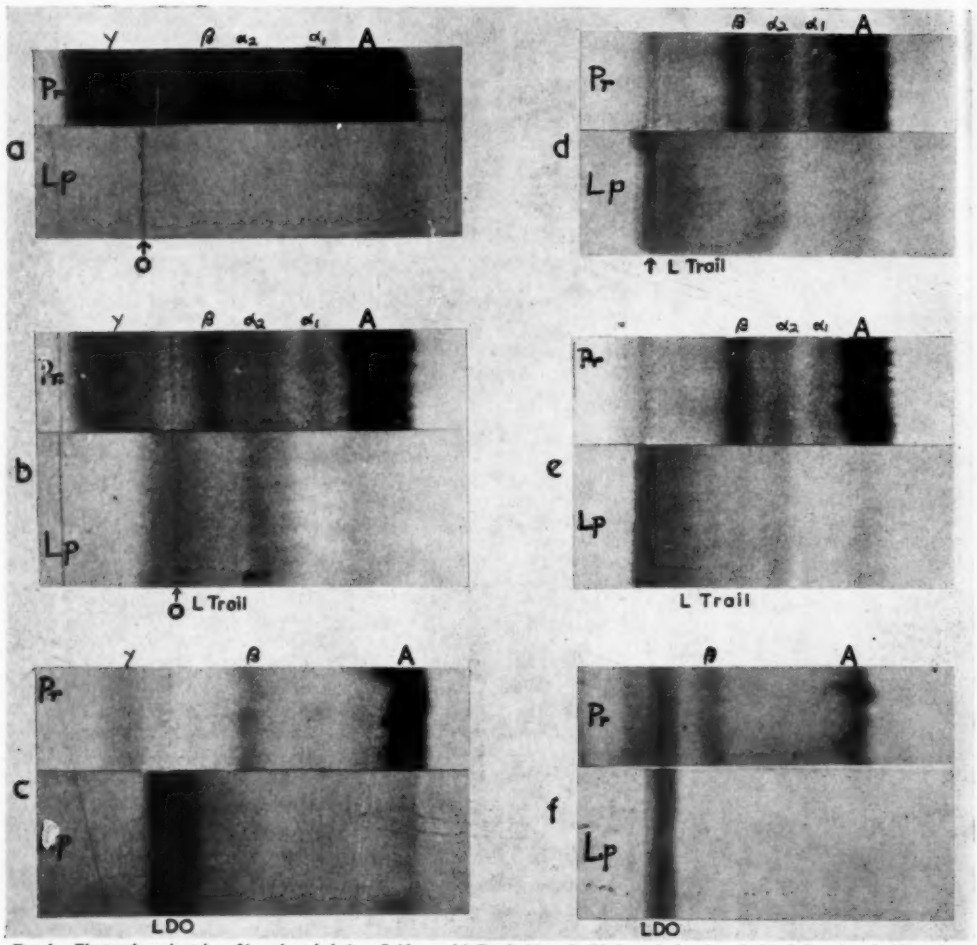

FIG. 1.—Electrophoretic strips of lymph and chylous fluids. (a) Cervical lymph, (b) thoracic duct lymph, (c) chylous lymph, (d) chylous lymph, (e) chylous ascitic fluid, (f) chylous urine.

Most of the fluids showed protein patterns related to that of serum and dependent on the total protein content, but the lipid patterns were less closely related to serum and dependent on the turbidity of the fluid as well as its total protein content.

The main classes of fluid examined will be considered briefly here.

Lymph.—Clear lymph from the cervical lymphatics gave protein patterns resembling those of serum, but the lipid patterns were either almost blank or showed a weak β lipoprotein band, perhaps partly due to contamination of the specimen with blood (Fig. 1a).

All seven specimens of thoracic duct lymph showed protein patterns resembling serum, but the lipid patterns were dominated by dense deposits of lipid at the origin and in the trail, which in some cases extended forwards as far as the α_2 globulin (Fig. 1b). In five samples a β lipoprotein band was present, but in four of these there was evidence of contamination with blood, so it is uncertain how much β lipoprotein was present in the uncontaminated lymph.

Chylous lymph from dilated inguinal and iliac lymphatics gave patterns (Fig. 1c and 1d) similar to those from thoracic duct lymph with a very dense lipid deposit at the origin and in two cases a moderately dense trail. In one sample some fat particles were so large that they separated as a cream at the top of the milky specimen when it was stored overnight in a refrigerator.

Clear Body Fluids.—The clear fluids (and a few turbid ones that cleared on ordinary centrifuging) showed patterns consistently related to the total protein content, which ranged from 0.4% to 4.9% (Fig. 2). In general the patterns fell into three groups:

(*a*) Specimens with a low protein content (oedema fluids, spermatocele and amniotic fluid) which, unless previously concentrated, showed only a definite albumin band and perhaps one or two faint globulin bands, β globulin usually being the most apparent; no lipid bands were visible (Fig. 2a).

(*b*) Fluids with an intermediate protein content (generally 2–4.5%) which showed the usual four or five protein bands but in altered proportions, and characteristically the α_2 globulin band was relatively weak; traces of lipid were sometimes visible but no definite β lipoprotein band (Fig. 2b).

(*c*) Fluids with a high protein content (4% or more) which gave protein and lipid patterns practically indistinguishable from those of serum (Fig. 2e, f, g).

Some of these clear fluids were centrifuged under water at 12,000 r.p.m. for one hour and examined in a focused beam of light. The lower layer scattered light strongly throughout its height, but the upper layer showed only very faint scattering indicating that only minute quantities of fat particles were present. A few of these fluids were treated with small quantities of dextran sulphate and calcium chloride in the manner of Burstein and Samaille (1958), when the pleural and ascitic fluids gave a precipitate of β lipoprotein but not the hydrocele fluids.

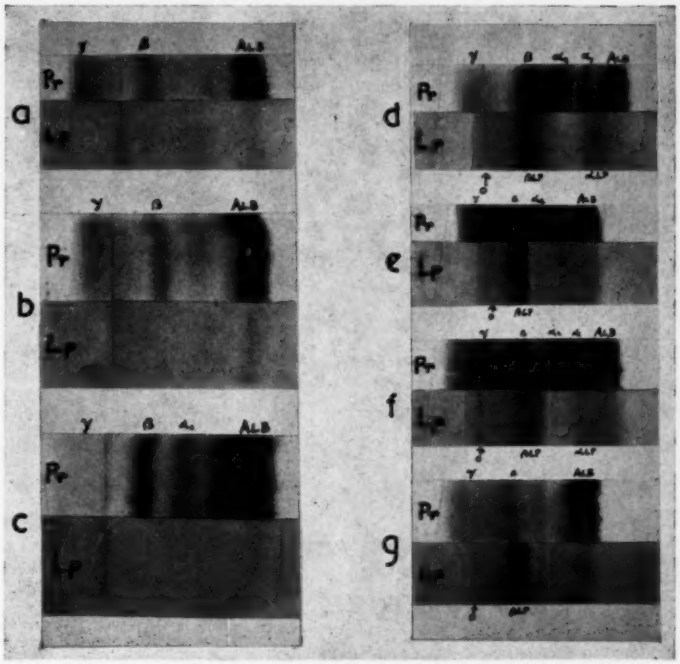

FIG. 2.—Electrophoretic strips of clear fluids. (a) Oedema fluid, (b) hydrocele fluid, (c) urine (nephrosis), (d) serum, (e) pleural fluid, (f) ascitic fluid, (g) bursa fluid.

One rare specimen that came into this survey was the fluid which had accumulated in a bladder that had been isolated surgically on account of a congenital obstruction of the bladder neck. This fluid contained 2.6 g.% protein, and although it was not viscous it produced a pattern with ill-defined protein bands and no lipid bands.

Nephrotic urines, although rich in protein, contained no lipid (Fig. 2c); the protein patterns generally showed little α_2 globulin, in striking contrast to the serum, where it was markedly increased. Two amniotic fluids showed only albumin, a faint β globulin, and no lipid bands.

Turbid Body Fluids.—Most of these were chylous fluids taken from patients with lymph-oedema. Seven specimens of chylous ascitic fluid (Fig. 1e) and one each of chylous pleural fluid and chylous blister fluid produced patterns similar to those given by thoracic duct lymph and chylous inguinal lymph. All showed a dense deposit at the origin, but there was considerable variation in the intensity and extent of the lipid trail. It was extremely difficult to tell from these patterns whether any β lipoprotein was present. Unfortunately these fluids were aspirated before

the centrifuge had been acquired. Two specimens of chylous ascitic fluid that had been kept refrigerated or frozen for some months were centrifuged, when most of the turbidity rapidly migrated to the top of an overlying water layer. The lipid separated in this way was washed with water and analysed and found to be mainly neutral fat with relatively small proportions of cholesterol (4% and 7%) and phospholipid (13% and 18%).

Five specimens of chylous urine were examined, including three from one patient taken during the last month of pregnancy. (This patient produced markedly chylous urine during the last few weeks of her third, fourth, and fifth pregnancies; the condition disappeared gradually during the fortnight following delivery.) In all specimens albumin and β globulin predominated in the protein pattern, and there was a dense deposit at the origin in the lipid pattern (Fig. 1f). A very short trail was seen with two specimens, but in no case was a β lipoprotein band visible. Two specimens were layered under water and centrifuged, when the lipid particles rapidly migrated to the top of the water. Separation and analysis of this lipid showed that it was mainly neutral fat with small proportions of cholesterol (3% and 1%) and phospholipid (1.5% and 7%).

A specimen that was turbid but not chylous was taken from a pseudomucinous cyst. This dark, viscous fluid cleared on centrifugation at 12,000 r.p.m. for two hours, the turbid material settling to form a brown deposit. The clear brown supernatant fluid gave a strip with ill-defined protein bands; there was a dense deposit of lipid at the origin and a considerable lipid staining level with the albumin.

Discussion

Traditionally, extravascular collections of fluid are divided into two classes: exudates with high protein content and transudate with low protein. Our collection of fluids showed no clear dividing line, and in fact continuous ranges of protein concentrations and patterns were found.

The lipid patterns fell into three groups:

(*a*) Almost blank patterns given by fluids containing little or no lipid, such as cervical lymph and most oedema fluids.

(*b*) Patterns similar to serum, usually showing a sharp β-lipoprotein band, given by clear fluids with a high protein content; these fluids were generally of neoplastic or inflammatory origin.

(*c*) "Chylous" patterns showing a dense deposit at the origin and a moderately dense "trail" and sometimes a lipalbumin; these were given by chylous fluids, thoracic duct lymph, and hyperlipaemic sera.

The lipid trail of chylous fluids is of particular interest; it is very probably due to a "spectrum" of particles migrating at diverse speeds, and probably of diverse sizes. They are presumably largely composed of neutral fat, and could be removed by centrifugation under water as with hyperlipaemic sera. When such a serum is centrifuged under water or saline much of the lipid responsible for the trail and deposit at origin in the original serum pattern migrates into the upper layer, and this layer on electrophoresis gives a very dense deposit at origin and usually only a very short trail (although with a few patients the trail is quite extensive).

The front of the lipid trail in patterns from chylous fluids is usually level with the α_2 globulin, but it is often ill defined and may vary in position with the details of electrophoresis or the amount of fluid applied. A similar "advanced front" to the β-lipid complex is seen in the serum patterns of patients with essential hyperlipaemia and in the lipaemia of uncontrolled diabetes (Wolff and Salt, 1958). Another condition in which the serum pattern shows an advanced front to the β-lipid complex is nephrosis, where a definite band can sometimes be discerned, a "pre-β lipid," and this band remains largely undissolved when a portion of the strip is extracted with ethyl alcohol before staining. In the case of chylous fluids and lipaemic sera the whole of the β lipid complex is extracted in alcohol, even its front edge. (An advanced β-lipid front is also given by stale sera.)

Smith (1957) has correlated for normal serum the paper electrophoretic components and the ultracentrifugal Sf groups of Gofman (de Lalla and Gofman, 1954). The deposit at the origin corresponds to particles with Sf values greater than 400 and the trail corresponds roughly to the Sf range 100–400; a lipid band, a "pre-β lipid," which in serum migrates between β globulin and the front of α_2 globulin, corresponds approximately with the range Sf 20–100. This correlation may not apply to lymph although there is no obvious reason why it should not do so. The presence of the origin and trail (presumably Sf 400 and Sf 100–400) lipoprotein groups in thoracic duct lymph suggests that this is a possible source of these groups in plasma and chylous fluids.

The conclusions that can be drawn from examination of paper electrophoresis strips must always be guarded, particularly where lipids are concerned. Although it is simple to produce strips giving a fair representation of the various proteins, considerably more skill and experience

are required to obtain a satisfactory comparative picture of the lipids. Nevertheless, carefully made strips give a more complete picture of the lipids than any other single method that can be carried out in the ordinary hospital laboratory. The difficulty in distinguishing between β lipoprotein and trail in chylous fluids may sometimes be overcome by high-speed centrifugation. Further, quantities of β lipoprotein too small to give a well-defined lipid band may sometimes be detected by dextran sulphate precipitation. Such a combination of methods offers a valuable means of lipid investigation.

Although the range of fluids examined here is quite limited, the following conclusions would seem permissible.

(1) A sharp β lipoprotein band will only be seen if the fluid has a high protein content and is clear.

(2) A chylous fluid gives a dense deposit at the origin and usually a lipid trail.

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TRUE GLUCOSE CONTENT OF LUMBAR AND VENTRICULAR CEREBROSPINAL FLUID

BY

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Glucose levels in 154 random specimens of lumbar cerebrospinal fluid, normal as regards cell and protein content, were estimated by the glucose-oxidase method and a mean value of 60.5 mg./100 ml. (S.D. 7.3) was obtained.

In 33 fasting patients subject to air encephalography the mean cerebrospinal fluid (C.S.F.) glucose level was 56.5 mg., and mean blood glucose level 65.5 mg. with an average C.S.F. glucose: blood glucose ratio of 0.85.

Estimation of the glucose content is a valuable adjunct to the enumeration and identification of cells and the determination of the protein content of cerebrospinal fluid (C.S.F.) as a diagnostic procedure. In the past, differences of opinion have existed as to the normal levels of glucose, and this has been in part due to the lack of a specific method for determining this substance in biological fluids. Recently methods utilizing glucose-oxidase have been employed for blood. Results of such estimations show that glucose is invariably present in smaller amounts than had previously been found using non-specific methods. This paper describes the normal ranges of C.S.F. glucose and compares them with glucose levels in venous blood.

Material

Glucose estimations were performed on 154 specimens of C.S.F. received in the laboratory. No preservatives were used, and estimations were completed within a few hours of the C.S.F. being drawn. Only fluids which were normal in all respects, such as cell count and protein, were used. These fluids were obtained at various times of the day and bore no fixed time relationship to food intake.

In addition 63 ventricular fluids were obtained at diagnostic ventriculography, usually under local anaesthetic (which often contained adrenaline), or immediately preceding major surgery. All these patients were in the fasting state. Cell and protein content was frequently normal, but slightly abnormal fluids, i.e., those containing less than 10 cells/mm. and 60 mg.% protein, were not excluded from analysis. Grossly abnormal fluids are not considered in this communication.

Thirty-three lumbar fluids, normal as regards cell and protein content, were obtained from patients subjected to diagnostic air encephalography. Most of these patients were not anaesthetized though heavily

TABLE I

GLUCOSE LEVELS, MEASURED BY GLUCOSE-OXIDASE AND FOLIN-WU METHODS IN C.S.F. AND VENOUS BLOOD*

Case No.	Cerebrospinal Fluid		Blood		C.S.F./ Blood Glucose Ratio
	Glucose-oxidase Method	Folin-Wu Method	Glucose-oxidase Method	Folin-Wu Method	
1	46	53·5	53	81·5	0·86
2	48	56	60	77	0·80
3	49	52	59	76	0·83
4	50	51	62	75	0·81
5	50·5	58	66·5	85	0·80
6	51	59	53	82	0·97
7	53		57		0·93
8	54	55·5	69	78	0·78
9	54·5	64	68	75	0·80
10	55	62	65	100	0·85
11	55	59	67	88	0·82
12	56	70	63	94	0·89
13	56	60	56	74	1·00
14	56·5	62	59	80	0·96
15	57	66	75	84	0·76
16	57	70	64	92	0·89
17	57·5	67	58	88	0·99
18	57·5	56	71	86	0·82
19	58	87	68	93	0·85
20	58	93	68	90	0·85
21	58	65	62	71	0·94
22	58	66	77	100	0·75
23	58·5		58		1·00
24	58·5	70	65	94	0·90
25	59		67		0·88
26	59	64	67	96	0·88
27	60	86	68	93	0·89
28	60	76·5	65	85	0·92
29	61	63	67·5	77	0·91
30	62	75	83	115	0·74
31	64	77	78	100	0·82
32	64	71	68	95	0·94
33	65	70	68	95	0·96
Mean	56·5	67·7	65·5	88·0	0·85

* Levels are expressed as mg. glucose per 100 ml. fluid.

sedated ; all were fasting. Immediately before lumbar puncture a sample of venous blood was drawn into a bottle containing 0.03 ml. of 8% formalin. Blood and C.S.F. were analysed by both the glucose oxidase and Folin-Wu methods.

Methods

Glucose was determined in blood and C.S.F. by the method previously described (Marks, 1959). "Glucose" was determined by a modified method of Folin and Wu (Harrison, 1947).

Results

Since the conditions under which the fluids in each group were collected differ so markedly, it is not possible to attach statistical significance to the observations in one group compared with those in another.

Results for true glucose estimations of 154 normal lumbar C.S.F. and 63 ventricular fluids are shown in Fig. 1. Mean glucose levels of 60.5 mg./100 ml. (S.D. 7.3) for lumbar and 61.2 mg./100 ml. (S.D. 11.3) for ventricular fluid were obtained.

Cerebrospinal fluid obtained at air encephalography from fasting patients showed a mean glucose level of 56.5 mg./100 ml. (S.D. 4.5), and the mean fasting blood glucose level was 65.5 mg./100 ml. (S.D. 6.4). The average ratio of glucose in C.S.F. to blood was 0.85 (S.D.=0.075).

In five patients, in whom the fluid was otherwise normal, ventricular glucose levels between 35 and 40 mg./100 ml. were found. Except in one case, a patient with a pituitary tumour and reduced blood sugar, no explanation for these apparently low levels is available. In C.S.F. from fasting patients true glucose was always lower than glucose estimated by the Folin-Wu method, by an average of 11.2 mg./100 ml. In a previous report (Marks, 1959) on a smaller group of patients who were not fasting an average difference of 5.5 mg./100 ml. was found.

Discussion

Although the propriety of considering C.S.F. from patients with disease of the nervous system as normal is questionable, the ethical difficulties in obtaining normal C.S.F. from healthy people has made it necessary to resort to considering C.S.F., normal as regards cell and protein content, as "normal" for the purpose of further analysis.

Katzenelbogen (1935), Lups and Haan (1954), and others have insisted that for the sugar content

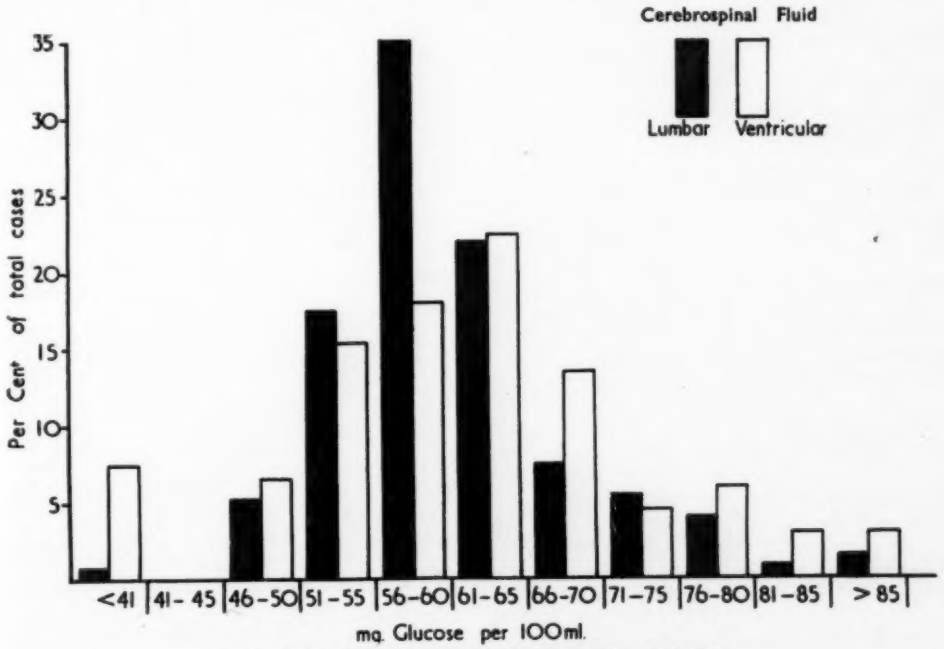

FIG. 1.—Distribution of true glucose in 154 lumbar and 63 ventricular fluids.

of C.S.F. to be of significance in diagnosis the fluid should be obtained from the fasting patient because they believe that it is directly related to the blood sugar level at any one time. However, Cumings and Carmichael (1934) in a series of experiments showed that slight elevations of blood sugar in the human subject following the administration of oral glucose were not accompanied by significant changes in the C.S.F. The present findings, that C.S.F. glucose levels in fasting and non-fasting patients do not differ greatly from each other, agree with these experimental results. Glucose elevations (and possibly depressions over a long period) are, however, attended by a proportionate change in the C.S.F. level of glucose.

I should like to thank Professor J. N. Cumings for his help and advice in the preparation of this paper.

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STANDARD BARIUM SULPHATE SUSPENSIONS

BY

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It has been shown that the state of the volumetric flask, the order of mixing the reagents, and the temperature of the reaction are important influences on the optical density readings of standard barium sulphate suspensions. The rate of mixing the reagents does not appear to be important.

A reproducible method for preparing a standard barium sulphate suspension with an optical density equivalent to 100 mg. protein per 100 ml. is described.

Standard barium sulphate suspensions have been used as standard opacities for (1) Maclagan's thymol turbidity test (1944) by Shank and Hoagland (1946) and Ley, Lewis, and Davidson (1946); (2) the zinc sulphate test described by Kunkel (1947); and (3) for the rapid fibrinogen test of Stirland (1956). Maclagan (1951) and Varley (1958), however, have experienced difficulty in reproducing the opacities of these suspensions, as has Ducci (1947), who first pointed out that a misprint in the article of Shank and Hoagland (1946) had caused the ensuing workers to use a 1% barium chloride solution (0.0962N) instead of a 2% solution (0.0962M), as first described by Wadsworth (1927). The former solution is the one now in general use. The experiments which we wish to report in summary support the views of Maclagan, of Varley, and of Ducci, but we have been able to define more clearly the conditions under which reproducible optical density readings can be obtained.

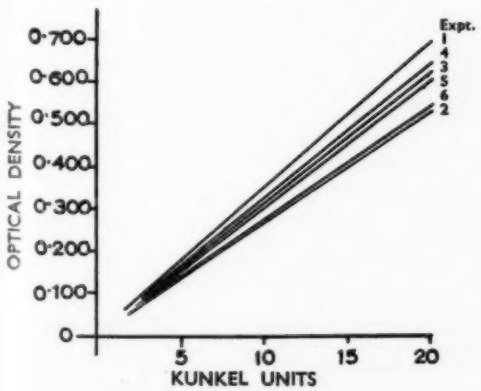

FIG. 1

Materials

1.15% $BaCl_2.2H_2O$ (AnalaR) solution.
0.2 N. H_2SO_4 (AnalaR).

Experiments and Results

Volume and Order of Mixing the Reagents.—Three millilitres of 1.15% $BaCl_2.2H_2O$ solution may be mixed with 97.0 ml. of 0.2 N. H_2SO_4 in many ways. After agitation, the optical density was read immediately on a "unicam" SP 600 spectrophotometer at 650 mμ, using a 10 × 10 × 45 mm. cuvette, against a control of glass-distilled water. The figure shows the mean results of six experiments. These are:

Experiment 1.—Dry flask+3.0 ml. $BaCl_2$ solution+made up to 100 ml. with 0.2 N. H_2SO_4. Mean O.D. of 19 results=0.702.

Experiment 2.—Flask rinsed with 0.2 N. H_2SO_4 and drained+3.0 ml. $BaCl_2$ solution+made up with 0.2 N. H_2SO_4. Mean O.D. of 15 results=0.538.

Experiment 3.—10.0 ml. 0.2 N. H_2SO_4+3.0 ml. $BaCl_2$ solution+made up with 0.2 N. H_2SO_4. Mean O.D. of two results=0.623.

Experiment 4.—20.0 ml. 0.2 N. H_2SO_4+3.0 ml. $BaCl_2$ solution+made up with 0.2 N. H_2SO_4. Mean O.D. of four results=0.648.

Experiment 5.—40.0 ml. 0.2 N. H_2SO_4+3.0 ml. $BaCl_2$ solution+made up with 0.2 N. H_2SO_4. Mean O.D. of two results=0.618.

Experiment 6.—80.0 ml. 0.2 N. H_2SO_4+3.0 ml. $BaCl_2$ solution+made up with 0.2 N. H_2SO_4. Mean O.D. of two results=0.549.

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Thus strict attention must be paid to the method of preparation of the standard suspension by mixing the reagents in a given order.

The Rate of Mixing.—Two 3.0 ml. pipettes were selected because of obvious differences in the sizes of the orifices at their tips. By using a standardized procedure it was shown that the rate of adding $BaCl_2$ solution to a 50.0 ml. pool of 0.2 N. H_2SO_4 and making up to the 100 ml. mark with 0.2 N. H_2SO_4 made no difference to the optical density reading.

Temperature of the Reaction.—The procedure was repeated, not only at room temperature (18° C.), but at 37° C., at 56° C., and at 100° C., the flasks with their contents being continually agitated during mixing, and then cooled thoroughly under running cold water so that the flask and contained fluid were restored to their normal dimensions before being filled to the 100 ml. mark with 0.2 N. H_2SO_4. The O.D. readings were 0.680, 0.640, 0.520, and 0.445 respectively at the above temperatures.

The temperature at which the reaction is carried out is as important as the order in which the reagents are added to the flask.

The Effect of Digestion.—The suspensions produced in experiments 1–6 above were digested at room temperature for 24 hours (Vogel, 1951), and the optical densities were read again after thorough resuspension. On the whole there was no consistency between the first and second readings.

Discussion

Our experiments show that there is great variability in the optical density readings, depending on the method employed. The two main sources of error are (1) the order of mixing the reagents, and (2) the temperature at which the main reaction is carried out. The reason for the variations is the simultaneous precipitation of barium chloride which is incorporated on the surface of the barium sulphate molecule (Vogel, 1951).

Protein graphs in constant use in this laboratory, and prepared by direct correlation of protein values with optical density values, show that the mean optical density reading for 100 mg. protein per 100 ml. at 650 mμ, i.e., 10 Maclagan units or 20 Kunkel units, is 0.656. The following method produces a standard barium sulphate suspension with this optical density.

Method of Preparing Standard Barium Sulphate Suspensions.—Take a dry 100-ml. volumetric flask. Place 50 ml. 0.2 N. H_2SO_4 in it and place this in a 37° C. water-bath until this temperature is reached by the sulphuric acid. While still in the bath, add 3.0 ml. of 1.15% $BaCl_2.2H_2O$ solution to the flask with gentle agitation. After 10 to 15 sec. remove the flask and cool it with its contents under cold running water, meanwhile continuing the gentle agitation. Once completely cool, top up to the 100 ml. mark with 0.2 N. H_2SO_4. Insert the stopper and invert the flask several times. Read the optical density of this barium sulphate suspension against glass-distilled water as a control in a "unicam" SP 600 spectrophotometer at 650 mμ.

Results.—The mean of 11 results is 0.663 (scatter 0.630–0.709), and this involves the use of two different batches of stock reagents. The scatter is now one-third of the overall scatter of the six experiments summarized in the figure.

Finally, the variabilities of standard barium sulphate suspensions described in this paper in no way influence the fundamental theories of the thymol turbidity test (Maclagan, 1944), the zinc sulphate test (Kunkel, 1947), or the rapid fibrinogen method (Stirland, 1956).

We wish to express our gratitude to Dr. A. D. T. Govan, Director of Research, for his encouragement and guidance during this research.

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TECHNICAL METHODS

Photographic Recording of Precipitin Bands in Agar Gel

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The method for producing antigen-antibody precipitin bands in agar gel, due to Ouchterlony, is becoming increasingly useful in diagnostic immunology as well as in research into antibody-antigen reactions. For both these purposes a dimensionally accurate record of all bands, including faint lines, crossovers, and spurs, is necessary for correct interpretation of the plate. The dimensional accuracy is obligatory if an investigation is to be carried out on the relative molecular weights of the proteins involved (Korngold and Van Leeuwen, 1957).

The method of drying the agar plate on to a lantern slide is not one of choice, as it is both laborious and the record is liable to deteriorate (Gell, 1955; Hayward and Augustin, 1957). Freehand drawings are both inaccurate and liable to bias. Most methods of photography used until now have employed the capacity of the precipitin band to reflect the incident light, making it necessary for the camera to be slightly inclined from the perpendicular to the plate to give an image of a well-lit band. This leads to dimensional inaccuracy, halation, and loss of definition, and grey bands and spurs are not recorded. Other disadvantages are that it is necessary to employ a very powerful light source a small distance from the plate, generating considerable heat, and a specially constructed apparatus and good recording camera, both expensive, are necessary.

This paper describes a method of photography giving high contrast, and definition, and dimensional accuracy which is both cheap and capable of being carried out without an advanced knowledge of photographic technique.

Materials and Methods

Petri Dishes.—Pyrex petri dishes, 7–12 cm. in diameter, are used. Any which show bubbles in the glass, scratches, or excessive convexity of the inside surface of the base are discarded, but it has been found that strain marks do not affect the photography. Each preparation must be inspected, as it has been found that bubbles form as the plates age. Those passing the inspection are washed, dried, rendered completely clean and grease-free in an acid cleaning mixture, and, after rinsing thoroughly with filtered distilled water, are dried inverted, in a completely dust-free atmosphere. From this point until the plates are poured great care must be taken that there is no contact between the fingers and the inside surface of the plate. The clean plates are stored in a dust-free container until used.

Preparation of the Agar.—Davis New Zealand agar at double concentration (2%) is prepared. This allows for the incorporation of a suitable buffer or salt solution at double the required strength. A phosphate-citrate buffer at a final ionic strength of 0.1, *p*H 7.6, was suitable for use with blood coagulation factors. A stock of agar is prepared by making 2 litres of 2% agar in distilled water, the agar being brought into solution by placing the container in a steam chest for one hour with occasional mixing by swirling. The solution is then filtered *twice* through a 20 cm. Büchner funnel containing one sheet of Green's "504½" filter paper covered by a 3 cm. thick layer of paper pulp with a thin layer of medium sand on it. The funnel and its contents are preheated by passing hot tap water through it for 20 minutes before filtration. A very gentle suction is applied (5–10 cm. Hg) to bring the agar through the funnel, at the same time avoiding boiling and subsequent froth. The first 20 ml. of fitrate is discarded. After the second filtration into the same flask the agar is distributed in 50 ml. amounts into clean, sterile bottles using a clean preheated measuring cylinder. The bottles are immediately stoppered and stored at 2–4° C. Neither bacterial nor fungal contamination or deterioration of the agar has been found over a six-month period of storage.

Agar Plates.—A flat, levelled board is used for the plates, and should be of such a size that a large relatively air-tight cover such as a glass tank or the lower half of a desiccator can be inverted over it to provide a dust cover and prevent undue evaporation of water from the agar. It has been found that it is not advisable to use another, larger, petri dish to cover each individual plate, as there is a tendency for condensed water to drop on to the agar and affect the development of the lines. The volume required to fill the plates to a depth of 3 mm. is calculated, and as the plates were of four standard diameters, lines of those lengths were drawn on the pouring board with the volumes of agar required inscribed on them so that the size of the plates could be rapidly checked without unnecessary handling. The plates are placed inverted on the board together with a clean 10 ml. "blowout" pipette with a wide jet (wider than B.S.S.).

A bottle of stock agar is placed in the steam chest and left for one hour to melt. During the time that the agar is melting an equal volume of the required buffer or salt solution is made up at double strength, containing 0.04% sodium azide as a preservative. This is then filtered twice through the same Whatman No. 1 filter paper into a 250 ml. Ehrlenmeyer flask standing in a boiling water-bath. The agar is removed from the steam chest and slowly poured through a funnel into the filtered buffer or salt solution, and the mixture swirled for a few minutes to ensure complete mixing. The flask is then removed

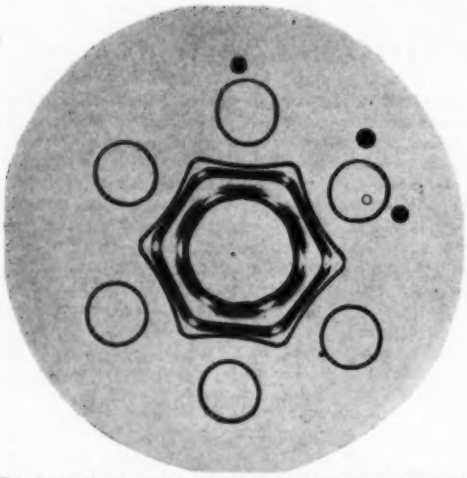

FIG. 1.—Ouchterlony plate demonstrating clarity obtainable by this method of photography. The plate was photographed after two days' development. The centre well contained the antiserum and wells 1 to 6 the antigenic solution. The black spot beside well 3 is dust and the circle in well 2 shows the appearance of an air bubble in the glass of the dish.

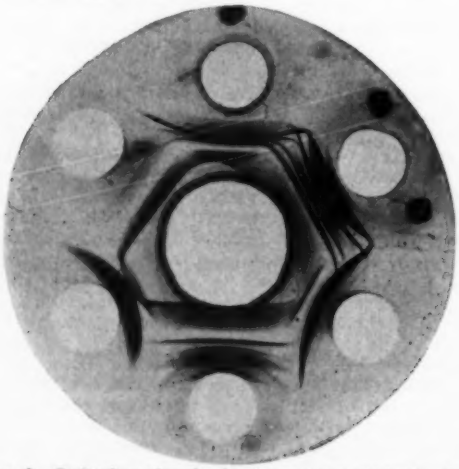

FIG. 2.—Ouchterlony plate showing the resolution obtainable. The background of this plate is dirty owing to bacterial growth, as this plate had to be developed for 10 days to show maximum development of lines. The experiment was concerned with the comparison of the antigens in two stages (A) and (B) of the preparation of a bovine clotting factor (antihaemophilic globulin). The antiserum had been prepared in a rabbit by intravenous injection of a fraction of bovine plasma rich in this factor. The centre well contained the antiserum. Outer wells 1, 3, and 5 contained the preparation A at increasing dilution and wells 2, 4, and 6 contained preparation B, likewise at increasing dilution. This plate shows the following patterns used in the interpretation of the results described by Wilson and Pringle (1955, 1956). Surrounding the centre well there is a "folded ribbon." Fusion of lines occurs between wells 1 and 2, and a "spur" from the third from centre line of well 2 joins two components of well 1, the line demonstrating a "crossover" as it passes to the outermost line at well 1.

from the boiling water-bath and the agar distributed into the plates, care being taken to apply very gentle suction to the pipette to avoid the formation of bubbles. The glass cover is then placed on the board and the plates left for about one hour to cool and gel. It is worth noting that a *p*H of below 6.5 or above 8 will probably interfere with the setting of the plates, as will autoclaving the agar at any stage or clearing with albumin. It has been found that the plates usually remain uncontaminated by bacteria or fungi for about a week after pouring.

The "Camera."—An ex-R.A.F. continuous processing unit enlarger (Fig. 3) was used, but the addition of a lamphouse to the back of a disused half- or whole-plate camera or projector will serve equally well. The lamp, a 75 watt "flashed opal" enlarger bulb, was adjusted to give a parallel beam of light incident on the petri dish. A large (15–20 cm.) diameter petri dish containing glycerol is supported on a screen having a circular aperture slightly larger than the agar plate and about 2 cm. above the condenser lens. Initially immersion oil was used to cancel out the refraction errors in the base of the plate, but as it had to be changed frequently owing to dust contamination this would have led to great expense, and glycerol was found to have a refractive index near enough to the glass to serve. The photographic plate was supported in a quarter-plate printing frame, having a mask with a circular aperture on the camera side (chiefly for aesthetic appeal in the finished prints) and a black backing plate to eliminate stray light reflected from the walls of the darkroom. The black sheet of metal at the top of the camera was to prevent reflection from the ceiling and was found to give a great improvement in contrast in the negative.

Photographic Technique.—The following materials are required for the preparation of the agar plates: Clean, dry, lint-free rag, filtered distilled water, lead shot, fine forceps, small spatula, filter paper, and a large petri or crystallizing dish as a receptacle for the agar. Photographic materials required are: Ilford N.40 $3\frac{1}{4} \times 4\frac{1}{4}$ process plates, Kodak white smooth glossy hard single weight paper (WSG 3S). Johnson's "M-Q" and "contrast" developers, and an acid hardening and fixing solution.

Method.—Lead shot is pushed into the agar above a predetermined point for identification on the final print. The dish is then inverted over the receptacle and the edge of the agar disk gently lifted with the spatula until the agar drops out of the petri dish, which is then carefully cleaned and placed on a clean filter paper. The disk of agar is carefully picked up and held between thumb and forefinger and washed on both sides with a stream of filtered distilled water from a plastic wash bottle. It is then returned to its dish, care being taken to avoid minute air bubbles under the agar, which is then covered with a 2–3 mm. layer of filtered distilled water. The prepared dishes are stood on filter paper, and covered with damp filter paper until photographed.

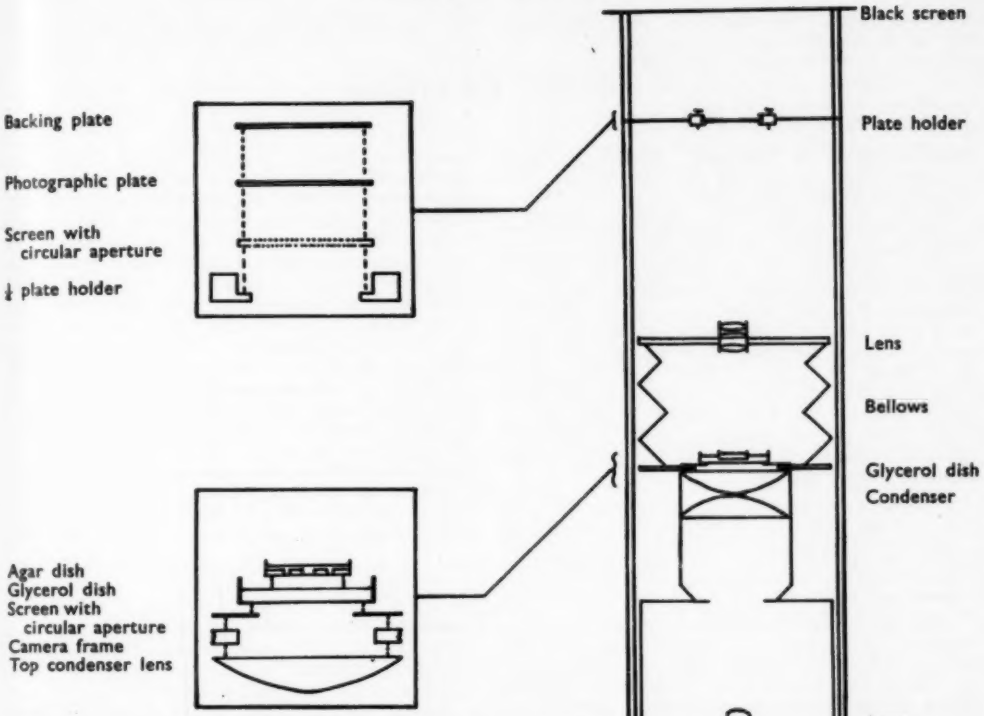

FIG. 3.—The camera is supported between two vertical strips of laboratory girder. The upper inset shows, in exploded view, the plate holding apparatus, and the lower inset shows an exploded view of the arrangement of the agar plate on the dish of glycerol supported over the condenser lens.

The dish is carefully lowered into the glycerol container in the camera, avoiding the formation of bubbles in the glycerol under the dish. The front of the camera is replaced and the image focused on a screen placed in the plate holder (a fogged, undeveloped photographic plate was found to provide the best screen). A photographic plate is then marked in one corner on the emulsion side with diamond with its serial number, and then substituted for the focusing screen, and the exposure made by switching on the "camera" light. Care must be taken to avoid vibration. A small aperture of *f*8–22 is recommended together with a neutral grey filter placed on the lens to give a controllably long exposure time of 5 to 10 sec. Shortening the exposure time for plates with only very faint bands does not increase the definition but only leads to printing difficulties. The exposure should be the same for every plate, regardless of the appearance of the bands.

The plates are developed for three minutes at 20° C., in Johnson's "M-Q" developer containing 0·5% wetting agent, constantly agitating the developing dish, fixed, and washed. It was found that a good criterion of correct exposure was that, if the plate was held 3 in. away from the red safelight after fixing, the difference between the edge of the lamphouse and the red screen was just sharply definable through the densest part of the negative. For most purposes the negatives were printed contact, and developed for about one minute in the "contrast" developer diluted one part with three parts of water, the development being stopped when the agar background just began to show. Any print developed in under 45 or over 75 seconds was discarded because faint lines and spurs were lost. The prints were fixed, washed, dried in a glazer, and mounted on paper for examination and filing. Lantern slides can be made by contact printing the plates.

I wish to thank Dr. Ethel Bidwell for her help and Dr. R. G. Macfarlane and Mrs. R. Augustin for their advice. The work was financed from a grant made to Dr. R. G. Macfarlane by the Medical Research Council.

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BOOK REVIEWS

Modern Trends in Pathology. Edited by D. H. Collins. (Pp. x+334+Index; 135 figures. 70s.) London: Butterworth. 1959.

The "Modern Trends" series has reviewed developments in some 20 medical subjects; several have had a second series and one even a third series, but it is only this year that *Modern Trends in Pathology* has appeared: Professor Collins must be congratulated on a work which will demand a second series quite soon. A list of the contributors includes the names of a large proportion of the most distinguished "morbid anatomists" of this country and two from the U.S.A., but the whole outlook of the book is quite unlike the old-fashioned "morbid anatomy," and the last paragraph of Sir Roy Cameron's chapter epitomizes this viewpoint.

> "Investigations such as these possess the merit of welding together structural and functional observations into a coherent story. And on this note I would wish to end, for I am convinced that the future of pathology lies precisely in this direction."

Although it may be invidious to single out separate chapters for mention, Sir Roy Cameron's "The Injured Cell," Pulvertaft's article on "Examination of Pathological Tissue in a Fresh State," Payling Wright's "Movements of Neurotoxins and Neuroviruses in the Nervous System," Campbell's "Pathological Relationships of 5-Hydroxytryptamine," Symington's "The Human Adrenal Cortex in Disease," and Collins' and Curran's "Pathological Ossification and Osseous Metaplasia in Man" have been to me a particular pleasure to read: but the best recommendation is that the reviewer, whose own daily work is in a somewhat different field, has read the whole book with great interest and stimulation throughout. All the chapters are concerned with actively developing aspects of pathology—and all leave the reader with the feeling that this is exciting work, none of it complete—and all in active development.

It is a book which should be in all libraries of pathology—and all young pathologists will receive very considerable benefit from its use.

E. N. ALLOTT.

Medical Laboratory Investigations: Their Use and Interpretation. By Ian Dawson and William Goldie; Foreword by R. J. V. Pulvertaft. (Pp. xv +260. 35s.) London: Butterworth. 1959.

The value of compendia of medical laboratory investigations varies with the uses for which they are intended. This book represents an attempt to introduce practising clinicians to the tools of the pathologist. It is quite clear that no senior clinician would consult such a book if he were investigating, say, a fluid and electrolyte balance which is dealt with in a very elementary fashion in three pages. For the junior clinician, however, this is a very brief summary of what he might find, the value of the tests with the normal ranges. On the whole, therefore, this book, taken section by section, is useful for the new house-physician, who must begin to learn in his first post how to use the laboratory and the pathologists. The danger of these books is that the young house-physician will end his interest in pathology at that stage. To prevent this it is essential, as the authors point out, that he should realize that there is no substitute for regular meetings and close co-operation between clinicians and pathologists throughout their years in the hospital. They must learn to use and to integrate each other's disciplines.

A. GORDON SIGNY.

Abnormal Haemoglobins: A Symposium organized by the Council for International Organizations of Medical Sciences (U.N.E.S.C.O.). Edited by J. H. P. Jonxis and J. F. Delafresnaye. (Pp. ix+427; 104 figures. 45s.) Oxford: Blackwell Scientific Publications. 1959.

In September, 1957, a symposium on abnormal haemoglobins, organized by the Council for International Organizations of Medical Sciences, was held. This volume reports the proceedings of the meeting, which are of the greatest interest to the biochemist, the geneticist, the haematologist, the general pathologist, the general physician, and the anthropologist. Our present knowledge of abnormal, genetically determined haemoglobins is reviewed both authoritatively and comprehensively.

Haemoglobinopathies clearly play an important role in general health and in infant mortality and malaria mortality rates in many countries. An ever-increasing number of these states is being reported, not only in African and Mediterranean races, but also in other peoples.

The book is well written and illustrated. A section headed "Conclusions" emphasizes that, although great progress has been made in our knowledge of haemoglobinopathies since Pauling and his colleagues discovered sickle-cell haemoglobin in 1949, a vast amount of work remains to be done. The bibliography and the index are comprehensive. This volume is strongly recommended to the wide variety of doctors and scientists interested in this field.

E. K. BLACKBURN.

A Laboratory Manual on Abnormal Haemoglobins. Prepared under the direction of J. H. P. Jonxis and T. H. J. Huisman for the Council for International Organizations of Medical Sciences and the Middle East Science Co-operation Office of U.N.E.S.C.O. (Pp. vii+39; 12 figures. 9s. 6d.) Oxford: Blackwell Scientific Publications. 1958.

This book contains in small compass the essentials for laboratory diagnosis of conditions which are characterized by abnormal haemoglobins. It will be of service not only to workers in this special field throughout the world, but, because of its clarity, to workers in general haematology. After an introductory section, physico-chemical techniques, including electrophoresis, and haematological studies are described. There are several minor errors of final proof correction on page 4 under "diagnostic methods," where page references are inaccurate. Although there is no index, references are particularly well placed at relevant sections. Two tables at the end of the book which show biochemical characteristics of the human haemoglobin types and clinical and haematological features complete a manual on which the authors are to be congratulated.

E. K. BLACKBURN.

The ABO Blood Groups: Comprehensive Tables and Maps of World Distribution. By A. E. Mourant, Ada C. Kopeć, and Kazimiera Domaniewska-Sobczak. (Pp. viii+276. 42s.) Oxford: Blackwell Scientific Publications. 1958.

The work embodied here was originally intended to be included in *The Distribution of the Human Blood Groups*, by A. E. Mourant, but so exhaustive a survey of the frequency of the ABO groups in the world population would have unduly increased the size of this book and moreover merited a volume of its own.

The authors have brought together all data published up to the end of 1957 on the frequency of the ABO blood groups (including A_1 A_2 BO groups) in world-wide human populations. This comprehensive information is presented in tables and maps, and includes figures and calculations which give the reader the opportunity of assessing for himself the reliability of the collected data.

There is a short introduction which, in addition to explaining the plan of the book, leaves the reader greatly impressed by the magnitude of the work and the meticulous searching of the literature so ably undertaken.

The material presented will greatly interest all blood-group enthusiasts and will be of much value to anthropologists and geneticists.

BARBARA DODDS.

Cancer of the Skin. By John C. Belisario; Foreword by A. C. Cipollaro. (Pp. xvii+321+Index; 201 figures. 50s.) London: Butterworth. 1959.

This is a valuable treatise on cancer of the skin which will appeal to the dermatologist, radiotherapist, and pathologist. It is well illustrated and contains 201 figures and eight excellent coloured plates. The author has worked in Australia, where the incidence of skin cancer, due mainly to the greater number of sunlight hours, is much higher than in Britain. He has personally treated patients, using radiotherapy and surgery when the occasion demanded, so he can write authoritatively on this aspect.

The term kerato-acanthoma is now the one most frequently used for lesions which may exhibit the clinical characteristics of rodent or squamous carcinomas and which may be benign and self-terminating. A chapter devoted to such lesions includes the most useful recent literature, and the author describes how an accurate microscopical diagnosis may not always be possible.

In the chapter on malignant melanomas, Dr. Belisario rightly insists on literal accuracy, and he proceeds to adopt a nomenclature which seems acceptable to the pathologist. While sarcomas of the skin are relatively rare tumours, little mention is made of leiomyosarcoma, but multiple idiopathic sarcoma of the Kaposi type is described in great detail.

This monograph will be well received by those concerned with cancer of the skin, in particular the practising dermatologist. The general pathologist will find it a valuable reference book, but in some chapters lack of histological details and staining techniques make it less useful for the specialist pathologist.

A. DICK.

Symposium on Nuclear Sex. Edited by D. Robertson Smith and William M. Davidson. (Pp. xiii+188; illustrated. 21s.) London: Heinemann. 1958.

In September, 1957, only eight years after the discovery of the sex differences in cell nuclei by Barr and Bertram, a symposium on nuclear sex was held in London at which Professor Barr gave the opening and concluding addresses. Especially in view of the remarkably rapid advances in this field, it is valuable to have this interim report as a record of expert opinion at that time. The 22 papers were read by authorities from Great Britain, Canada, U.S.A., Europe, and Israel.

The papers are produced verbatim and the discussions on them are printed in full. The editors, being the chairman and secretary of the organizing committee, have published a readable account and have included a full bibliography on each paper. The book is divided into three sections with an adequate contents list but no index, and the quality of the production, including the illustrations, is excellent. Even the amateur (perhaps aided by a glossary from elsewhere) will find it pleasant reading. In the foreword, Sir Robert Platt remarks that this was one of the most exciting and enjoyable congresses he ever attended, and its spirit is so well conveyed in this book that the reader will join him in gratitude to the organizers.

The book cannot be too highly recommended to the clinical pathologist with an interest in histology.

A. G. MARSHALL.

Polymyositis. By John W. Walton and Raymond D. Adams. (Pp. x+270; 47 figures. 32s. 6d.) Edinburgh and London: E. & S. Livingstone. 1958.

The concept of an acute or subacute polymyositis, occurring alone or associated with such diseases as scleroderma, dermatomyositis or rheumatoid arthritis, is well recognized. The present monograph is an attempt to broaden this concept so as to include a variety of more chronic conditions, conditions which are otherwise extremely difficult to classify. This attempt is based on a personal series of 40 cases, 17 derived from a retrospective analysis of biopsy material. Of the 17 patients thus studied, nine still survived at the time of the investigation. This monograph derives much, too, from the wide experience of the authors in the experimental investigation of muscle disease.

Their definition of "polymyositis," because of its controversial nature, merits quotation. The condition (p. 184) may affect patients of either sex and of any age. Typically, the presenting symptom is weakness of the girdle and proximal limb muscles, although any muscle may be affected. There may be associated malignant disease. The condition may be fatal, may remit spontaneously, and may respond to cortisone. The muscle lesions include necrosis, vacuolar or granular degeneration, and phagocytosis: areas of regeneration are also found. Fibres vary in size, and there may be central migration of muscle-cell nuclei. Perivascular and interstitial infiltrations by inflammatory cells are seen. The breadth of this definition will be immediately apparent to the diagnostic pathologist, and indeed it is further emphasized by the statement that patients may not experience pain and tenderness in the muscles; and by the fact that in four of the 35 cases investigated histologically there was no cellular infiltration to establish the presence of an inflammatory lesion.

The authors are aware of the terminological and diagnostic difficulties implicit in so broad a concept of polymyositis. Indeed, among the most valuable features of their monograph is the clarity with which they have reviewed the literature and analysed the classification and nomenclature of muscle disease. In particular, their account of the differential diagnosis between chronic polymyositis and progressive muscular dystrophy is likely to be of great practical value. Some will perhaps question their claim that in the diverse and often chronic manifestations of polymyositis there is a single underlying disease process, related to that of the other collagen diseases. On the other hand, a detailed well-documented and well-illustrated monograph of this kind, in which the emphasis throughout is on the natural history of the disease, is bound to be most welcome to all those who are interested in the pathology of skeletal muscle.

W. ST. C. SYMMERS.

OBITUARY

R. B. H. GRADWOHL

Rutherford Birchard Hayes Gradwohl, who died on May 9, 1959, at St. Louis, Missouri, at the age of 82 years, was born in Baltimore, Maryland, his father having emigrated there from Strasbourg in 1855. Although his original ambition was to enter the United States Naval Academy, after his parents moved to St. Louis he chose medicine as a career and in 1898 graduated from Washington University School of Medicine. He began his internship at St. Louis City Hospital, and here showed the qualities which were to be the foundation of his life and career, an enthusiastic interest supported by untiring energy to improve his own knowledge especially in relation to pathology and pathological facilities. Thus in 1898 he was pressing for improvement in the laboratory facilities in the St. Louis City Hospital with success, and in 1899 he was working with Dr. A. Ravold on the bacteriological problems of the city water supply, which were not unconnected with the Chicago Drainage Canal. Having saved sufficient money, in 1900 he set out for Europe, stopping on his way to visit Sir William Osler in Baltimore. After studying pathology and chemistry for a short time at Heidelberg he obtained the position of volunteer assistant to Professor R. Langerhans in Berlin, and while there attended the classes of Virchow. He finally returned to St. Louis after spending a year at the Pasteur Institute in Paris. He was then appointed to a teaching position in bacteriology and pathology at the Marion Sims-Beaumont Medical School, later to become St. Louis University, and eked out his salary of 25 dollars a month by becoming one of the coroner's necropsy physicians.

Apart from taking an active part in the improvement of medico-legal facilities throughout the U.S.A. and developing the crime laboratory in St. Louis, he was the original convener of a meeting in St. Louis which resulted in the formation of the American Academy of Forensic Sciences in 1948. He is probably, however, best known for his book *Clinical Laboratory Methods and Diagnosis* which was first published in 1936, although in 1914 he had written a book on blood and urine chemistry in association with Mr. J. Blaivas, and in 1932 another on *Laboratory Technique* with his wife and niece. He also translated Schilling's *Das Blutbild*. Gradwohl founded the first school for training laboratory technicians in 1920, a remarkable example of his foresight.

It was perhaps most significant that when he returned home from Berlin on his first visit he brought back with him a copy in his own handwriting of the inscription over the entrance to Virchow's necropsy room and that he still possessed it at his death. "Hic Locus Est Ubi Mors Gaudet Succerrere Vitae."

F. E. CAMPS.

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THE EFFECT OF HEPARIN ON THE EARLY STAGES OF BLOOD COAGULATION

BY

J. R. O'BRIEN

From the Central Laboratory, Milton Road, Portsmouth

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From experiments reported it is concluded that heparin combines with and inactivates Christmas factor (C.F.) to form reversibly a heparin-C.F. complex. Apart from the effect of heparin on C.F. and on thrombin, heparin in moderate concentrations was not shown to inactivate any other coagulation factors.

" Available " heparin is defined as heparin in such a state that it can delay some clotting systems. Heparin was rendered " unavailable " or inactive by plasma C.F. and especially by serum C.F., by platelet protein (but not intact platelets), and by platelet-like activity of serum (P.L.A.S.). These three proteins uniquely among all the plasma and serum proteins inactivate heparin.

If an appropriate concentration of heparin is added to whole blood the clotting time is prolonged, due presumably to the inactivation of C.F. by heparin: " available " heparin disappears during clotting and none is found in the serum. The interactions summarized above probably account for these findings. The disappearance of available heparin would also account for the normal thrombin generation and prothrombin consumption observed when heparinized blood clots.

Some properties of P.L.A.S. are reported and its possible role in normal coagulation is considered. Heparin is known to become attached preferentially to one protein rather than another. Some plasma and serum fractions can be arranged in order of their increasing affinity for heparin thus: β Lipoproteins < thrombin clotting system < C.F. < platelet protein < protamine sulphate.

Heparin is used extensively in clinical medicine. Mast cells contain heparin, and these cells have been described in the subendothelial layers of the blood vessels (McGovern, 1955): thus heparin may have some physiological anticoagulant role. Precise knowledge of its anticoagulant action may therefore be of practical importance. The antithrombic action of heparin has long been known, but its action on the earlier stages of coagulation is less clear (e.g., Walton, 1955). Biggs, Douglas, and Macfarlane (1953) showed that heparin depresses intrinsic thromboplastin formation. Douglas (1956) suggested that heparin inhibits clotting early in the coagulation process. O'Brien (1958a) claimed that heparin inactivated Christmas factor (C.F.). Shanberge, Barlas, and Regan (1958) claim that the heparin antagonist, protamine sulphate, also inactivates C.F., although Hougie (1958) does not agree. The antiheparin activity of platelets was reported by van Creveld and Paulssen (1951). Garrett and Klein (1957) showed that lipid-extracted platelet material, presumably a protein, contains the antiheparin activity, while the lipid extract contains the clot-accelerating lipid of platelets.

Methods

Native Platelet-rich Plasma.—This was prepared by taking venous blood by syringe and centrifuging 5 ml. lots at 500 r.p.m. for five minutes and collecting the supernatant. This was used at once or stored on ice. Silicone-coated apparatus was used throughout.

Native Platelet-poor Plasma.—This was prepared by centrifuging native blood at 4,000 r.p.m. for seven minutes and removing two-thirds of the plasma and re-centrifuging this. Silicone-coated apparatus was used throughout.

Citrated Plasma.—This, platelet-rich or platelet-poor, was prepared as above, but the blood was first mixed with one-tenth of its volume of 3.8% trisodium citrate solution in water.

Normal Serum.—Normal serum was prepared from normal blood by allowing it to clot in contact with glass for at least one hour and centrifuging at 4,000 r.p.m. for 10 minutes and collecting the supernatant.

Platelet-poor Serum.—This was prepared from native platelet-poor plasma by allowing it to clot in contact with glass, centrifuging, and collecting the supernatant.

Absorbed Plasma.—This was prepared by adding to plasma a tenth of a volume of aluminium hydroxide

gel of such a strength that, after five minutes' incubating and removal of the aluminium hydroxide by centrifugation, the plasma was suitable for the thromboplastin generation test, i.e., the "prothrombin time" was 1–4 minutes. This procedure removes C.F. and some other proteins.

Absorbed Serum.—Absorbed serum was similarly prepared from normal serum.

Cephalin Suspension.—A cephalin suspension was made by suspending 1 mg. of asolectin in 1 ml. of water or saline.

Heparin.—This was "pularin" brand appropriately diluted with saline; its strength is recorded in units where 100 u. is equivalent to 1 mg. of heparin.

Thrombin.—Lyophilized human thrombin prepared by the Lister Institute was dissolved in saline so that 1 ml. contained 5 units of thrombin. A weak thrombin solution contained 1 unit per ml.

Fibrinogen.—Ampoules of lyophilized human fibrinogen prepared by the Lister Institute contained 1.37 mg. of protein. This was dissolved in 1 ml. of water and 5 ml. of saline for use in the thrombin clotting test, and in 50 ml. of saline for the thrombin generation test.

Tests for "Available" Heparin

Lengthening of Plasma Clotting Time.—The recalcification clotting time of platelet-poor citrated plasma in the presence of powdered glass was measured. If a mixture containing heparin added to this plasma delays the clotting time, available heparin is present.

Lengthening of Thrombin Clotting Time.—Heparin co-factor is present in all the plasma and serum fractions used. One volume of the plasma under investigation to which heparin had been added and one volume of fibrinogen were mixed and allowed to warm in a 37° C. water-bath for one minute. One volume of a strong thrombin was added and the clotting time was determined. If this clotting time was longer than that when plasma alone was substituted for the heparinized plasma, then available heparin was present. The degree of lengthening of the clotting time was related to the amount of heparin present. This system was found to be very sensitive to calcium ion concentration.

Ability to Inactivate Heparin.—The relative ability of two plasmas to inactivate heparin was compared by adding a similar critical quantity of heparin to both plasmas. How much of the added heparin remained "available" was estimated by carrying out thrombin clotting tests. The plasma giving the longer thrombin clotting time still contained more "available" heparin and therefore had inactivated less.

Tests for Christmas Factor Activity

The usual test was the thromboplastin generation test (Biggs and Douglas, 1953) in which normal dilute absorbed plasma and cephalin or platelets were used and the test serum diluted 1 in 10 was added.

In some situations the shortening of the clotting time of plasma from a patient suffering from Christmas disease was used.

Tests for Platelet-like Activity of Serum

The test for this activity, reported in 1955 (O'Brien), was copied exactly. Equal volumes of the serum under investigation and of normal citrated platelet-poor plasma are mixed and placed in a water-bath at 37° C. for one minute and then recalcified. This tube is watched till it clots, and thereafter 0.1 ml. aliquots are transferred at minute intervals to a series of tubes containing 0.4 ml. of fibrinogen solution. The clotting time of fibrinogen measures the concentration of thrombin in the original mixture at the time the subsample was taken. The shortest fibrinogen clotting time, measuring the maximum thrombin concentration, is directly related to the P.L.A.S. content of the serum.

Tests for Cephalin Activity of Platelets.—(*a*) The thrombin generation test was carried out exactly as in the estimation of P.L.A.S., but with the substitution of the platelets under investigation for the serum.

(*b*) The Stypven time of platelet-poor plasma is greatly accelerated by the addition of isolated cephalins or by cephalin bound in a macromolecule such as platelet protein (O'Brien, 1958b). Equal volumes of plasma, dilute Russell viper venom, and the platelets under test were recalcified and the clotting time recorded.

Results

Action of Heparin on Christmas Factor.—This was studied using the thromboplastin generation test (Table I, part 1). It may be concluded that heparin in the appropriate strength prevents the formation of thromboplastin but does not inactivate formed thromboplastin, and that heparin in the concentration used does not affect the clotting system in the subsample tubes.

TABLE I

HEPARIN AND CHRISTMAS FACTOR IN THE THROMBOPLASTIN GENERATION TEST

Dilute Absorbed Plasma and Cephalin in All Runs and Serum Diluted 1 in 10:	Time Elapsed (min.)*	Clotting Time (sec.)*
Normal serum + saline	3	11
Normal serum + heparin 0·5 u./ml. before recalcification	3	19
Normal serum + heparin 0·5 u./ml. 3 min. after recalcification	4	12
Normal serum + saline	3	9
Heparinized serum + saline	5	29
" " + 0·1 ml. normal serum	3	9
" " + 0·1 ml. Christmas disease serum	3	24
Heparinized serum + protamine sulphate (0·004 mg./ml.)	4	9

*Time elapsed is the time from recalcification till maximal thromboplastin formation is demonstrated by the shortest clotting time in any subsample tube, recorded here as "clotting time."

The abnormal thromboplastin generation test in the presence of heparin was corrected by the addition of normal serum, but not by an excess of absorbed plasma or Christmas disease serum or by absorbed normal serum. "Heparinized serum" was prepared by letting heparinized blood clot and was shown (vide infra) to contain no available heparin. Its behaviour in the thromboplastin generation test is summarized in Table I, part 2. The pre-incubation of heparinized serum with a strong concentration of platelets also revealed C.F. activity (O'Brien, 1959). It is concluded that heparin combines with C.F. and inactivates it. Protamine sulphate and platelet protein remove the heparin from the C.F.-heparin complex to reveal C.F. activity again.

Action of Christmas Factor on Heparin.—Christmas-factor-containing and C.F-deficient plasmas and sera were mixed with critical quantities of heparin and the remaining available heparin activity was determined by carrying out thrombin clotting times. Platelet-poor native plasma and "platelet-poor serum" was used (except as indicated); thus the concentration of Ca++ was constant throughout, and any effect due to platelets or P.L.A.S. was excluded. From Table II, A and B, it will be seen that the thrombin clotting times of mixtures containing C.F. were shorter than those containing none. It may be concluded that plasma C.F. had inactivated some heparin and that serum C.F. inactivated more heparin than plasma C.F. This might be due to the fact that C.F. in serum is a Ca-C.F. complex (Bergsagel, 1955). Normal platelet-poor serum was accordingly decalcified by mixing it with one tenth of its volume of 3.8% trisodium citrate for 24 hours. This citrated serum was compared with another aliquot of the same serum citrated immediately before testing (Table II C). The decalcified serum apparently inactivated considerably less heparin. Thus the ability of serum C.F. to inactivate more heparin than plasma C.F. is probably related to the fact that serum C.F. is a Ca-C.F. complex.

Serum from platelet-poor Christmas disease blood contains factor VII: absorption of this serum will remove it. Neither of these two kinds of sera inactivated appreciable quantities of heparin. It is concluded that factor VII does not inactivate a demonstrable amount of heparin.

Action of Platelets on Heparin.—Platelet-rich and platelet-poor native plasmas inactivated the same small amount of heparin. Thus intact platelets do not inactivate heparin but fragmented platelets (Table III A) inactivate much heparin. These findings are fully compatible with the previous reports of, for example, Wolf (1956) and Rapaport and Ames (1957).

Action of Heparin on Platelets.—The cephalin activity of platelets in platelet-rich plasma was measured in the thrombin generation test. To an aliquot of this plasma was added sufficient

TABLE II

THE INACTIVATION OF HEPARIN BY C.F.

	Contains	Thrombin Clotting Time (sec.)				
		+ Saline	Heparin (u./ml.)			
			0·125	0·25	0·5	1·0
A						
Native normal plasma ..	Plasma C.F.	24	31	42		
Absorbed native normal plasma	—	26	64	76		
Citrated normal plasma*	Plasma C.F.	25	50	66		
Christmas disease citrated plasma*	—	25	59	80		
B						
Normal serum	Serum C.F.	16			16	21
Absorbed normal serum	—	16			21	>60
Christmas disease ..	—	15			40	>60
Absorbed Christmas disease serum	—	15			39	>60
C						
Citrated normal serum ..	C.F.	20		40	>60	
Normal serum citrated ..	Ca-C.F.	20		24	38	
Citrated Christmas disease serum	—	20		40	>60	
Christmas disease serum citrated	—	19		40	>60	

* These two plasmas were one month old when tested.

One volume of platelet-poor plasma or serum was used throughout and one volume of fibrinogen was added when sera were compared. One volume of saline or heparin in the strength indicated was then added and finally one volume of weak thrombin in A and normal strength thrombin in B and C, and the clotting time recorded.

TABLE III

INACTIVATION OF HEPARIN BY C.F., PLATELETS, AND P.L.A.S.

	Contains	Thrombin Clotting Times (sec.)		
		Saline	Heparin (u./ml.)	
			0·25	0·5
A. Citrated platelet-rich plasma in silicone	Intact platelets	12	65	>60
Citrated platelet-rich plasma agitated	Platelet fragments	12	29	>60
B. Normal serum	C.F., P.L.A.S.	12·5	20	24
Platelet-rich absorbed serum	P.L.A.S.	12·5	22	>60
Platelet-poor serum ..	C.F.	12·5	30	>60
Platelet-poor absorbed serum	—	12·5	>60	>60
C. Citrated normal absorbed serum	P.L.A.S., no Ca	18	30	90
Absorbed normal serum citrated	P.L.A.S.	18	24	50

To one volume of the material in column one was added one volume of saline or heparin in the strength indicated and one volume of fibrinogen. Thrombin was then added and the clotting time in seconds is recorded.

heparin, e.g., 0.5 unit/ml., together with powdered glass so that the clotting time was not much altered. The thrombin generation curves of the untreated and heparinized samples were closely similar. (With excess heparin, e.g., 1.0 unit, the amount of thrombin generated was less.) The Stypven time of fragmented platelets in plasma was not lengthened by the addition of a moderate excess of heparin. It is concluded that the cephalin activity of platelets is not impaired by heparin. (Yet heparin is inactivated by platelet protein.)

Garrett and Klein (1957) report that the extracted platelet lipid is responsible for thromboplastin formation while the lipid-extracted, water-soluble, presumably protein, moiety exhibits antiheparin activity. Snellman, Sylvén and Julén (1951) point out that heparin and dextran sulphate are attached to lipoproteins not through the phospholipid but that the linkage must be achieved by the polypeptide and may be dipolar in type. It seems therefore that platelet activity in clotting is due to cephalin groups and these are active whether they are " free " or bound to the platelet protein, and that this activity is not influenced by heparin. The protein moiety, whether combined with or freed from cephalin, is responsible for inactivating heparin, the antiheparin effect (see O'Brien, 1958b).

Action of P.L.A.S. on Heparin.—From Table III B it will be seen that serum containing P.L.A.S. (platelet-rich absorbed serum) inactivates more heparin than serum containing no P.L.A.S. (platelet-poor absorbed serum). The long clotting time of recalcified citrated heparinized platelet-poor plasma (11½ minutes) was hardly affected by the addition of absorbed platelet-poor serum which contains no P.L.A.S. (9½ minutes): but the addition of absorbed normal serum rich in P.L.A.S. gave a short clotting time (3½ minutes). It is concluded that P.L.A.S. inactivates much heparin.

The effect of decalcifying P.L.A.S. on its ability to neutralize heparin was tested. Absorbed serum citrated for 24 hours was compared with absorbed serum citrated immediately before testing (Table III C). It is concluded that decalcified P.L.A.S. inactivates less heparin.

Other Coagulation Factors.—The total amount of heparin inactivated by whole blood or by normal serum was almost entirely accounted for by the three interactions reported above ; namely, those involving plasma and serum C.F., platelet protein, and P.L.A.S. Thus no other plasma or serum fractions inactivate appreciable amounts of heparin. Conversely it was shown that heparin in appropriate concentration had no apparent effect on the coagulant activity of Hageman factor, antihaemophilic globulin, factor V, factor VII, and platelets.

The Action of Heparin on the Clotting of Whole Blood

(1) Appearance of Antiheparin Activity during Clotting of Normal Blood.—Two rows of tubes were prepared containing 0.1 ml. of two appropriate strengths of heparin, e.g., 0.5 u./ml. and 0.25 u./ml. To all tubes was added 0.4 ml. of fibrinogen solution. Twenty millilitres of freshly drawn blood was placed in a test tube in the water-bath and allowed to clot and at appropriate intervals 2 ml. aliquots were removed, citrated, and centrifuged: one hour later 0.1 ml. of each aliquot was transferred to the tubes containing the fibrinogen and heparin and mixed, 0.1 ml. of a strong thrombin solution was added, and the clotting times recorded (Table IV). Initially the blood inactivated very little heparin since long thrombin clotting times were recorded. The ability of the blood to inactivate heparin increased slightly up to the time of the appearance of the first signs of clotting in the blood and thereafter increased rapidly and markedly during the next five minutes.

TABLE IV

APPEARANCE OF ANTIHEPARIN ACTIVITY DURING NORMAL COAGULATION

Time after Taking Blood (min.)	Thrombin Clotting Time in secs. with:		
	Heparin (0·4 u.)	Heparin (0·2 u.)	Saline
0	67	30	18
4	68	32	
7	53	28	
10	60	26	
14	55	24	
→18	30	21	
22	24	19	
26	23	20	
40	23	20	

At the times indicated 2 ml. of blood was removed and citrated: one hour later 0·1 ml. of the centrifuged plasma was added to 0·4 ml. of fibrinogen and 0·1 ml. of saline or heparin and thrombin added. → Indicates first visible clotting.

(2) Disappearance of Heparin Activity during Clotting of Heparinized Blood.—Twenty millilitres of blood from either heparinized volunteers, e.g., 2,000 u. heparin injected i.v. five minutes before bleeding, or normal blood subsequently heparinized, e.g., 0.2 u./ml. blood, was placed in a beaker at 37° C. and powdered glass added to accelerate the clotting time. The blood usually clotted in about 15 minutes. In each run several of the following tests were carried out on subsamples removed from the beaker at appropriate intervals up to one hour.

(*a*) *Available platelet cephalin* was monitored up to the time of clotting only (thereafter the platelets are largely caught up in the clot) by serially removing

1 ml. subsamples from the beaker, citrating, and estimating the Stypven time one hour later. This test was shown to be unaffected by the concentration of free heparin present.

(*b*1) *Thrombin formation* was monitored by subsampling 0.1 ml. amounts into 0.4 ml. of fibrinogen solution.

(*b*2) Thrombin generation could also be indirectly studied by doing two-stage prothrombin tests on the citrated subsamples of the blood one hour later.

(*c*) *The Formation of P.L.A.S.*—Amounts, each of 1 ml., were removed and citrated. They were centrifuged and (usually) absorbed to remove platelet activity and any free heparin, and one hour later they were tested for P.L.A.S. as previously described.

(*d*1) *The neutralization of heparin by the blood* in the beaker was monitored by subsampling into a fibrinogen solution, adding a strong solution of thrombin and estimating the thrombin clotting time.

(*d*2) The neutralization of heparin was further studied by estimating the clotting time of platelet-poor citrated normal plasma to which was added hour-old citrated subsamples of the blood from the beaker.

Similar appropriate observations were carried out on unheparinized blood.

Results

The rate of formation and the approximate concentrations of platelet cephalin, of thrombin, and of P.L.A.S. were not appreciably influenced by the initial presence of heparin in the blood (Fig. 1). Prothrombin was completely consumed 10 minutes after clotting. Tests (*d*1) and (*d*2) revealed an initial slow fall and then a rapid fall and complete disappearance of "available heparin" about the time visible clotting occurred. Indeed, apart from some variation in the time scale, the heparinized blood gave, along the parameters studied, similar results to those of normal blood. When 1 unit of heparin per ml. was added to blood the clotting time was lengthened from 10 minutes to four hours. If powdered glass was added the clotting time was still somewhat prolonged and no thrombin generation could be demonstrated (it was probably formed too slowly), but prothrombin consumption was complete 15 minutes after clotting and available heparin disappeared. When sufficient heparin was added to render the blood incoagulable for 24 hours apparently all the heparin remained available at this time.

It is concluded that a moderate dose of heparin delays clotting by its action on C.F. The subsequent steps in the coagulation process are little altered; in particular thrombin generation is normal. This must be due to the absence of "available" heparin at this time. Before fibrin forms, some heparin is presumably inactivated by plasma C.F. and the increasingly available platelet protein. The sudden disappearance of all heparin activity is presumably due to the formation of serum C.F. and P.L.A.S., both of which inactivate much heparin.

The Nature and Significance of P.L.A.S.—The preceding experiments have demonstrated some properties of P.L.A.S.: its origin and nature and function will now be briefly considered (Table V). It is formed during clotting only if platelets are

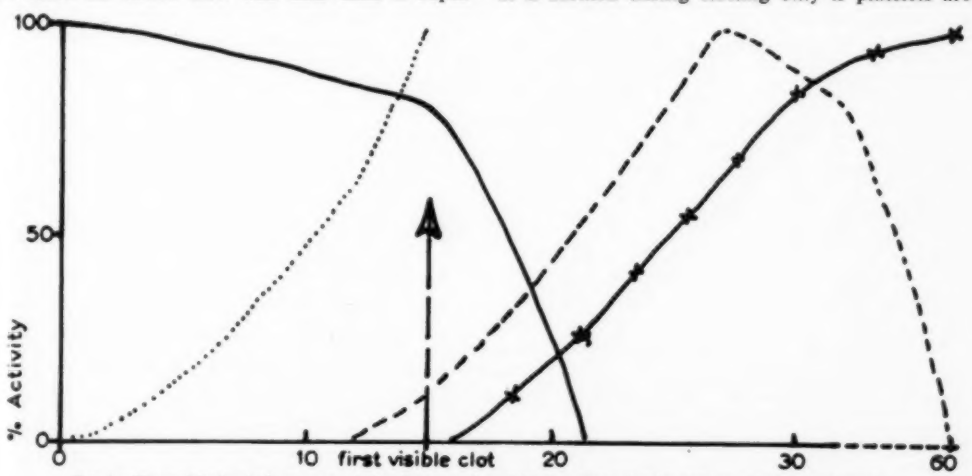

FIG. 1.—The activity level of some coagulation factors and available heparin during the clotting of heparinized blood. Platelet cephalin . . . . , thrombin – – – – , P.L.A.S. X—X—X, available heparin ——.

TABLE V
SOME PHYSICOCHEMICAL AND COAGULATION PROPERTIES OF P.L.A.S. AND PLATELETS COMPARED

	Absorbed by $Al(OH)_3$ Gel	With Native Platelet-poor Plasma			Inactivates Heparin	Inactivated by Heparin
		Accelerates Stypven Time	Thrombin Generated	P.L.A.S. Formed		
	1	2	3	4	5	6
(a) Absorbed platelet-rich serum (P.L.A.S.)	No	No	Yes	—	Yes	No
(b) Cephalin ..	Yes	Yes	Yes	No	No	No
(c) Platelet fragments	Yes	Yes	Yes	Yes	Yes	No
(d) Absorbed platelet-poor serum (no P.L.A.S.) ..	No	No	No	—	No	No

present: when added to plasma it has the same effect as platelets in permitting thrombin formation: and, like platelet protein, it inactivates heparin. It is thus likely, at least in part, to be derived from platelets. Unlike platelets it is not absorbed by aluminium hydroxide gel and it does not accelerate the Stypven time. Platelet-like activity of serum therefore almost certainly has no available cephalin groups, and it is soluble, and yet it permits thrombin generation. Platelet-like activity of serum may be a degradation product of platelets after they have exerted their activity in the clotting processes. The evidence is also compatible with the concept that the platelet protein-cephalin complex is altered to P.L.A.S. with the loss of "available cephalin" groups and that P.L.A.S. then plays an essential part in blood coagulation.

Relative Ability of Some Proteins to Bind Heparin.—Platelet protein, P.L.A.S., and C.F. appear to be unique among all the plasma and serum proteins in their ability to bind heparin strongly. It is remarkable that only coagulation factors should have this attribute. Since these three proteins uniquely share this property it is probable that some part of their molecular structure must also be similar; P.L.A.S. and serum C.F. are probably combined with calcium and all three may contain lipid (O'Brien, 1956, and unpublished work). At present there is no further information.

The results of the foregoing experiments can also be considered from the point of view of the relative ability of the various coagulation factors to bind heparin. Heparin in small quantities added to plasma is bound largely by the β lipoproteins (Bernfeld, Donahue, and Berkowitz, 1957: Nikkilä, 1953), yet this heparin is "available" to delay the thrombin clotting system. This latter system must have a greater affinity for heparin than the β lipoproteins. Conversely, heparin bound to C.F. does not influence the thrombin clotting system. The affinity of heparin for C.F. must be greater than its affinity for the relevant protein involved in the thrombin clotting system. The addition of platelet protein to the heparin-C.F. complex liberates C.F. activity, indicating that the affinity of heparin for platelets is greater than that for C.F. And the ability of protamine sulphate to reverse the heparin effect on all these factors indicates that the attraction between heparin and protamine sulphate is greatest of all. This conclusion may be expressed diagrammatically:

Increasing Affinity for Heparin

$$\beta\ \text{Lipoprotein} < \begin{matrix}\text{Thrombin}\\ \text{Clotting}\\ \text{System}\end{matrix} < \text{C.F.} < \begin{matrix}\text{Platelet}\\ \text{Protein}\end{matrix} < \begin{matrix}\text{Protamine}\\ \text{Sulphate}\end{matrix}$$

My thanks are due to Mrs. J. Cheater, A.I.M.L.T., for excellent technical assistance. This work was carried out during the tenure of a grant from the Medical Research Council for technical assistance which I gratefully acknowledge.

Addendum

Since the preparation of this article, Grieg (*Brit. med. J.*, 1959, **2**, 25) has reported that he can confirm the basic experiments with heparin and C.F. reported here and elsewhere (O'Brien, 1958a).

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OBSERVATIONS ON THE EFFECT OF MAGNESIUM ON BLOOD COAGULATION

BY

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Previous observations on the effect of oral magnesium and peptone on thrombin generation time are examined. The smallest amounts of magnesium and peptone regularly producing a significant shift demonstrable after one hour are 0.25 g. magnesium and 15 g. peptone.

The oral effect can be reproduced by the intravenous injection of magnesium. The minimum amount required is 50 mg. The effect is transient, and after 30 minutes can no longer be demonstrated.

Amounts of magnesium of the same order as those acting on intravenous injection can produce a shift of thrombin generation time *in vitro*. This action of magnesium *in vitro* can be antagonized by the addition of calcium.

Small amounts of magnesium added to fresh unclotted human plasma prolong the clotting time considerably. This action was shown to be due to an antagonism between Mg^{++} and Ca^{++}, both competing for combination with clotting factors (Greville and Lehmann, 1944). Such ion antagonism had been demonstrated in muscle and in nerve end-plates where the breakdown of adenosine triphosphate is activated by Ca^{++} in muscle and by Mg^{++} in end-plates (Greville and Lehmann, 1943). The amount of magnesium required to demonstrate this effect *in vitro* was about 10 times the normal plasma level, and considerably greater than could be practically achieved *in vivo*. Attempts to test the action of magnesium *in vivo* were, however, made, notably in Germany, where, during and shortly after the 1939–45 war, magnesium sulphate was widely used as a muscle relaxant. It was noted that the blood of some patients examined *post mortem* after such treatment had remained unclotted. Schnitzler (1957) has summarized these experiences in his report on the use of 200 mg. of magnesium given three times daily, post partum, to prevent thrombosis. We have not been able to confirm his results, which indicated a highly significant prolongation of the whole blood clotting-time after a single dose of 300 mg. of magnesium. Malkiel-Shapiro, Bersohn, and Terner (1956) claimed that they improved the clinical state of patients with coronary disease when they administered magnesium parenterally. This they related to an effect of the magnesium on the coagulation mechanism. Brown, McGandy, Gillie, and Doyle (1958) were unable to find such a relation between magnesium and lipids in health or in disease. Anstall, Huntsman, Lehmann, Hayward, and Weitzman (1959) described the effect of the oral administration of 1 g. magnesium on the clotting processes. Peptone had to be given with the magnesium to increase its absorption. This was thought to be due to the effect of amino-acids on the solubility of sparingly soluble salts of divalent cations, as demonstrated by Lehmann and Pollak (1942). Glutamic acid was also effective. To demonstrate the effect it was necessary to use the whole blood thrombin generation test (Macfarlane and Biggs, 1953), rather than the clotting time (Lee and White). A delay in thrombin generation of at least four minutes was obtained in successful experiments one hour after the administration of 1 g. Mg^{++} and 50 g. peptone. Both had to be given together, and neither could produce this effect alone. It was not always possible to demonstrate a rise in the serum magnesium concentration in otherwise successful tests.

The present paper describes attempts to elucidate the following aspects of this effect:

(1) The minimum amount of orally administered magnesium and peptone required to produce a shift in thrombin generation time;

(2) the relation between the effect obtained by orally administered magnesium and that obtained by intravenous injection ;

(3) the relation between the effect obtained by intravenous injection and that exerted by the addition of magnesium to fresh blood *in vitro*.

Experimental Procedure

Macfarlane and Biggs's thrombin generation test (1953) was performed as previously described (Anstall *et al.*, 1959). For the work *in vitro*, 5 ml. of blood was drawn into a siliconed syringe already containing either 0.1 ml. of water or the salts under investigation dissolved in a constant volume of 0.1 ml. of water.

Results

Varying the Quantity of Oral Magnesium and Peptone.—The same effect obtained with 1 g. of magnesium and 50 g. of peptone could also be produced with 0.25 g. of magnesium and 15 g. of peptone, but, whereas with the first amounts the shift in thrombin generation could be demonstrated for five to six hours, with the second amounts it was present after one hour, but could no longer be found after two hours. Lowering the magnesium dose below 0.25 g. yielded equivocal results, regardless of how much peptone was given ; and lowering the peptone below 15 g. yielded, with some individual constancy, negative results in some, and equivocal or positive results in other subjects. Table I shows results of a typical series of experiments performed on one individual at intervals of four to seven days.

TABLE I
EFFECT OF ORAL MAGNESIUM AND PEPTONE ON THROMBIN GENERATION TIME

Oral Dose (g.)		Thrombin Generation Time (min.)		
Magnesium Citrate (g. Mg)	Peptone (g.)	Before Dose	One Hour after Dose	Shift
0·5	50	10	15½	5½
0·5	30	12	18	6
0·25	30	13½	17½	4
0·25	15	12½	18	5½
0·25	7·5	13	15	2

Injection of Magnesium.—Blood was withdrawn first to determine the basal time of thrombin generation. Afterwards, magnesium sulphate or magnesium chloride was injected intravenously in a volume of 1 to 2 ml. Blood was withdrawn five minutes later to allow for a thorough distribution of the magnesium. A shift in thrombin generation of four to five minutes' duration was found regularly after the injection of 50 mg. Mg^{++}. Some effect could be found 15 minutes after the injection of 50 mg., but it had vanished after 30 minutes. No significant shift was obtained after the injection of 25 mg. of the ion.

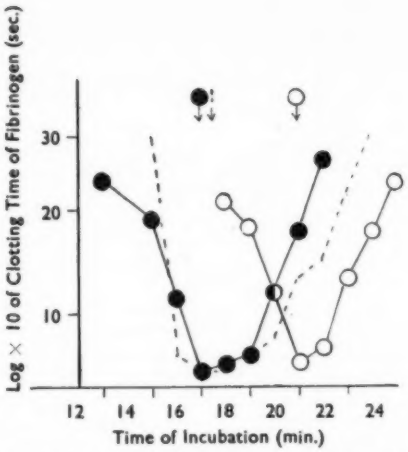

FIG. 1.—Whole blood thrombin generation before, 5 min. after, and 30 min. after intravenous injection of 50 mg. of magnesium ion. Peak of thrombin generation before injection 17 min., 5 min. after injection 21 min., and 30 min. after injection 17½ min. ●——● Before, O——O 5 minutes after, and ---- 30 minutes after injection of 50 mg. Mg^{++}.

The transient nature of the change after the intravenous injection is in marked contrast to the action of the orally administered magnesium and peptone (1 g. and 50 g. respectively), lasting for six hours. It is evident that, once absorbed, the effect of magnesium on clotting is rapidly neutralized, and it would, therefore, appear that the slow absorption after taking magnesium by mouth may be therapeutically advantageous. Judging from the rapidity with which the effect of intravenously administered magnesium diminishes, it might be assumed that even after only five minutes a decay in activity may already have occurred. Thus, it is possible that were this decay not so pronounced (or, as in oral dosage, if the magnesium slowly and continuously enters the blood stream), a much smaller quantity might have a significant effect. This could account for the frequent failure to demonstrate a rise in serum magnesium in otherwise successful experiments.

Experiments *in Vitro*.—Table II shows two typical experiments demonstrating the effect of small amounts of magnesium added to fresh-drawn blood. The amount of magnesium added *in vitro* was of the same order as those which

TABLE II

EFFECT *IN VITRO* OF MAGNESIUM^{++} ON THROMBIN GENERATION DEMONSTRATION OF Mg^{++} Ca^{++} ANTAGONISM

Experiment	$MgCl_2$ (mg. Mg^{++})	$CaCl_2$ (mg. Ca^{++})	Thrombin Generation Time (min.)	
			Peak	Shift
I			16	
		1·5	24	8
		0·5	16½	½
	0·15		25	9
	0·15	0·5	18	2
	0·15	0·1	19	3
			17	1
	0·15		24½	8½
II			23	
	0·3		37½	14½
		0·5	21	−2
	0·3	0·5	26	3
			24½	1½

Blood samples were taken at half-hourly intervals from two individuals. Each 5 ml. of blood was added in the syringe to 0·1 ml. of water or salt solution.

had produced a shift in thrombin generation *in vivo*. Equivalent amounts of potassium chloride and potassium sulphate had no effect. The fact that magnesium at such concentrations caused a shift in thrombin generation *in vitro* suggests that the action *in vivo* is directly on the blood, rather than intermediately on some other organ or system in the body.

Table II also illustrates another aspect of the magnesium effect. We had previously suggested (Anstall *et al.*, 1959) that Mg^{++} acted by competing with Ca^{++} when it was added to a clotting system in which Ca^{++} was in its optimal concentration. Large amounts of Ca^{++} added to blood are, of course, known to inhibit clotting. Furthermore, Mg^{++} will antagonize Ca^{++} when this ion is at its optimal concentration. If Ca^{++} is below its optimal concentration, Mg^{++} may actually activate the process which is catalysed by Ca^{++}, since on taking its place in that process it will not replace available Ca^{++} but complement the insufficient amount of catalyst available. These relationships have been set out in detail by Greville and Lehmann (1943, 1944), whose work took its origin from the contradictory results reported on the effect of Mg^{++} on the adenosine triphosphate splitting activity of myosin, some workers having described an activation, others an inhibition. Since then Born (1956) has reported that platelets have a high adenosine triphosphate content which falls rapidly on clotting, and Bettex-Galland and Lüscher (1959) have described an actomyosin-like protein prepared from platelets.

It was found that 0.5 mg. of Ca^{++} added to 5 ml. of blood had no significant effect on thrombin generation. This quantity was, therefore, considered suitable for demonstrating any possible antagonism of Ca^{++} on the effect of Mg^{++} (see Table II). It will be seen in Table II that 0.5 mg. of Ca^{++} by itself did not influence the thrombin generation time but that it considerably shortened the shift produced by 0.15 mg. or 0.3 mg. of Mg^{++}. These results link the present work with earlier observations of Greville and Lehmann (1944), who, however, used larger amounts of both ions and tested the whole plasma clotting time.

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A SIMPLIFICATION IN THE ESTIMATION OF "PROTHROMBIN TIME" FOR THE CONTROL OF ANTICOAGULANT THERAPY

BY

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A modification is described which reduces considerably the amount of pipetting involved in the estimation of "prothrombin time."

The procedure used by the authors is described in full. Particular attention is paid to the preparation and storage of the thromboplastin and to the sterilization of the citrate tubes used for the collection of specimens.

During the last few years the number of patients on treatment with anticoagulant drugs has shown a steady increase, which is likely to be maintained. The simple, one-stage prothrombin estimation of Quick using dried brain is usually considered to be adequate for the control of anticoagulant therapy (cf. Medical Research Council Working Party, 1959) and it has been in use in this laboratory for a number of years. The technique employed here has been essentially that described by Biggs (1951), except for the use of 0.02M instead of 0.025M calcium chloride and, more recently, phenol-saline solution in the preparation of the thromboplastin extract (Powell, 1957). It seemed that a considerable saving of time might be effected by the elimination of some of the tedious pipetting involved in the standard procedure. This could be done by mixing in bulk the thromboplastin extract and calcium chloride solution and blowing the plasma into 0.2 ml. of this mixture (instead of mixing plasma and thromboplastin and blowing in the calcium chloride). In view of the prevalent assumption that preliminary mixing of plasma and thromboplastin is necessary, a series of plasma samples was tested by both methods.

Soon afterwards it came to our notice that a similar modification had been proposed by Montigel and Pulver (1952). They prepared their mixed reagent by suspending a tablet of thrombokinase "Geigy" in calcium chloride solution, and a thrombokinase-calcium reagent is now commercially available in tablet form (Geigy). However, many workers may wish to be independent of commercial sources of supply, since the necessary reagents are easily prepared.

This paper first presents the experimental results supporting the proposed modification, and then describes in full the procedure employed in this laboratory (Appendix).

Material and Methods

Blood was collected in citrated tubes (see Appendix) from patients under treatment with anticoagulants (in most cases "dindevan"* but in some "sinthrome"*) and from normal people. The plasma was separated by centrifuging and placed in another tube, from which portions were taken for testing. First, the test was done in the usual way, 0.1 ml. of plasma being mixed with 0.1 ml. of diluted thromboplastin extract (see Appendix), the mixture left in the water-bath at 37° C. for 30 seconds and then 0.1 ml. of 0.02M calcium chloride (previously warmed to 37° C.) blown in. For the proposed modification, 0.2 ml. of a mixture of equal volumes of 0.02M calcium chloride and diluted thromboplastin extract was placed in the tube, which was left in the water-bath for 20 to 30 seconds. The plasma, previously kept at room temperature, was then blown in and the clotting time was noted in the usual way (see Appendix). Over the range 15° to 30° C. the temperature of the plasma did not significantly influence the results obtained. Care was taken to perform both tests on a given specimen within a few minutes of each other, so as to obviate errors that might arise from changes in the plasma on standing. Results were related to a normal value obtained by precisely the same technique, as described in the Appendix, and expressed as a "prothrombin clotting time ratio."

Results

The results of the comparison are summarized in the Table, from which it will be seen that there is a close correlation between the two procedures.

*Dindevan is phenylindanedione. Sinthrome is 3-[2-acetyl-1-4-nitrophenyl]ethyl]-4-hydroxycoumarin.

TABLE
COMPARISON OF PROTHROMBIN CLOTTING TIME RATIOS BY STANDARD PROCEDURE AND PROPOSED MODIFICATION

Standard Procedure	Proposed Modification (Mean and Observed Range)	No. of Plasma Samples Compared
1·0	1·1 (1·0–1·1)	3
1·1	1·1 (1·0–1·2)	11
1·2	1·2 (1·1–1·3)	12
1·3	1·3 (1·2–1·4)	13
1·4	1·4 (1·3–1·5)	12
1·5	1·5 (1·3–1·6)	22
1·6	1·6 (1·5–1·7)	16
1·7	1·6 (1·5–1·8)	25
1·8	1·7 (1·6–1·9)	11
1·9	1·8 (1·7–2·1)	19
2·0	1·9 (1·7–2·1)	18
2·1	2·0 (1·9–2·1)	19
2·2	2·1 (1·9–2·3)	11
2·3	2·2 (2·0–2·4)	17
2·4	2·3 (2·1–2·5)	12
2·5	2·4 (2·2–2·5)	2
2·6	2·4 (2·3–2·5)	4
2·7	2·6 (2·3–2·8)	5
2·8	2·7 (2·5–2·9)	4
2·9	2·8	1
3·0	3·2	1
3·1	2·9 (2·8–3·0)	2
3·2	3·1 (3·0–3·4)	5
3·3	3·2	1
3·4	3·5	1
3·5	3·3 (3·2–3·5)	2
3·8	3·5	1
3·9	3·7 (3·6–3·7)	2
4·0	3·5	1
4·4	4·0	1
4·6	4·5 (4·3–4·6)	2
4·8	4·5	1
5·0	4·6	1
5·2	5·1	1
5·7	6·2	1

There is a tendency for the simplified procedure to give slightly lower ratios with the more prolonged times.

Fig. 1 illustrates the stability of the thromboplastin-calcium reagent.

Discussion

These experiments clearly show that the proposed modification is reliable and does not give misleading results. In only one instance was there a serious discrepancy, but it is thought that an error in manipulation had occurred since a specimen of blood obtained from the same patient a few days later gave results in good agreement by both methods.

The tendency of the modified procedure to give slightly lower ratios with the more prolonged times is no disadvantage as long as this is appreciated. It is, indeed, recognized that different procedures give different ratios (Powell, 1957) and it is essential to know which procedure a laboratory employs before deciding on the level to aim at in therapy. According to Biggs (1951), the therapeutic range of prothrombin clotting-time ratios in the control of anticoagulant therapy is 1.5 to 2.5, i.e., for the technique she describes. Others recommend a range of 2.0 to 2.5 (cf. M.R.C. Working Party, 1959). This latter range would correspond to about 1.9 to 2.4 by the simplified procedure described in this paper. The difference is, in any case, slight and probably is not more than one might expect to find between the results of one laboratory and another, or sometimes even between two people working in the same laboratory.

This procedure, unlike that of Montigel and Pulver, does not involve warming the plasma to 37° C. before mixing it with the thromboplastin-calcium reagent. Furthermore, addition of the plasma to the thromboplastin-calcium reagent, rather than vice versa, appears slightly more convenient in that it permits distribution of the reagent among a number of tubes with a single pipette graduated at 0.2-ml. intervals.

APPENDIX

Method

Preparation and Storage of Brain.—A human brain is stripped of the membrane and superficial blood vessels, washed with water and then macerated with

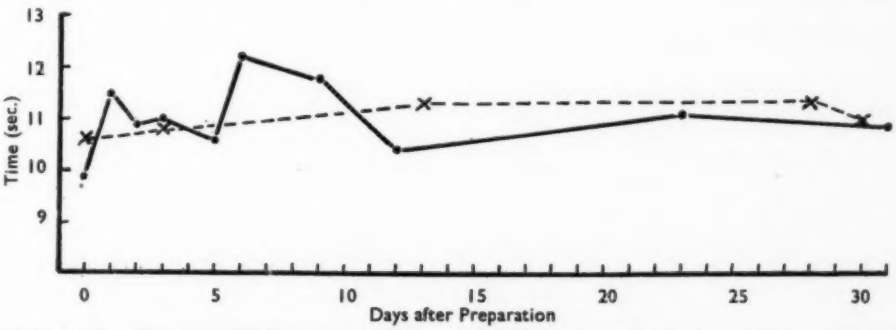

FIG. 1.—Stability of thromboplastin-calcium reagent when stored in the refrigerator: normal values obtained with two preparations over a period of a month.

acetone in a mortar. (The cerebellum is best excluded on account of the difficulty of removing completely the membranes and blood vessels.) The supernatant fluid is decanted and replaced with fresh acetone and the maceration continued, this procedure being repeated until a friable mass is obtained. The tissue is sufficiently dehydrated when a small portion, removed from the mortar and allowed to dry, can be crumbled to a dry powder. The acetone is then decanted and the brain is spread on large sheets of filter paper to absorb the excess of solvent and allowed to dry. Finally the dried brain, after thorough mixing, is placed in jars and stored in vacuum desiccators containing phosphorus pentoxide and kept in the refrigerator. (The main stock of brain is stored in one desiccator and a smaller amount in another from which it is weighed out as required. In this way the main stock of material is not exposed unnecessarily to air, moisture, and changes in temperature.)

Preparation of Thromboplastin Emulsion.—Dried brain, 2 g., is extracted at 37° C. for 15 minutes with 20 ml. of 0.85% w/v aqueous sodium chloride containing 0.5% w/v phenol, and stirred at five-minute intervals. After a few hours' standing to allow visible particles to settle, the supernatant fluid is removed ("stock thromboplastin extract"). This should be stored in the refrigerator but not frozen solid. The optimum concentration of thromboplastin for routine use is determined as follows. Normal blood is collected in a citrated tube (see below). The plasma is separated and diluted with four volumes of 0.85% w/v sodium chloride. Then 0.1 ml. of the diluted plasma is mixed with 0.1 ml. of stock thromboplastin extract, the mixture is incubated at 37° C. for 30 seconds, 0.1 ml. of 0.02M calcium chloride is blown in, and the time taken for clotting is noted. The test is repeated with dilutions of stock thromboplastin extract in phenol-saline (e.g., 1+1, 1+2, and so on), and the dilution giving the shortest clotting time is selected ("diluted thromboplastin extract"). A portion or all of this diluted thromboplastin extract is then mixed with an equal volume of 0.02M calcium chloride solution for routine use ("thromboplastin-calcium reagent"). This mixture, stored at 4° C. to 8° C., retains its potency for several weeks (Fig. 1).

Preparation of Citrated Tubes.—Amounts, each of 0.2 ml., of a 3.8% w/v aqueous solution of sodium citrate dihydrate (stored in the refrigerator) are measured into tubes (75 × 10 mm.) marked at exactly 2 ml. The tubes are then securely stoppered with rubber bungs and are autoclaved at 15 lb. for 20 minutes. It is convenient to autoclave the tubes in stainless steel racks stacked on top of one another and held together by rods, the top layer of tubes being covered by a metal plate: in this way the bungs are kept in place and no air enters when the tubes are allowed to cool. After autoclaving, each tube is labelled with a "sterile" label bearing the date of preparation and covering the bung. Tubes are issued to the wards and out-patient department with instructions that they be stored in an upright position.

Collection of Blood.—Blood is collected in the usual way and the tube is filled exactly to the 2 ml. mark. The plasma is separated by centrifugation.

The Test.—Thromboplastin-calcium reagent, 0.2 ml., is placed in a small test-tube (about 60 × 10 mm.), which is then left in the water-bath at 37° C. for 20 to 30 seconds. Citrated plasma at room temperature, 0.1 ml., is blown in, a stop-watch being started at the same time. The tube is slanted, and rotated gently about its long axis until the first appearance of fibrin strands. These are seen clearly if the tube is held underneath a light and against a dark background. The test is performed in duplicate at least. (When the test is repeated the tube is kept in the water-bath to within about five seconds of the time indicated by the first estimation.) Normal plasma specimens are tested in the same way and the result obtained with the patient's plasma is expressed as a ratio, viz.:

$$\text{Prothrombin clotting time ratio} = \frac{\text{Clotting time observed with patient's plasma}}{\text{Average clotting time observed with normal plasma}}$$

Notes on the Method

Preparation and Storage of Dried Brain.—These are essentially as described by Aggeler, Howard, Lucia, Clark, and Astaff (1946), with slight modifications. In our experience the dried brain, stored as described, retains its potency for at least a year.

Standardization of Thromboplastin.—The method is that of Biggs (1951), except for the use of phenol-saline.

Thromboplastin Extract.—The use of phenol to preserve emulsions of fresh brain was suggested by Toohey (1950); and Powell (1957), working in this laboratory, showed that extracts of dried brain can be preserved in the same way for at least two weeks. Powell's findings can be confirmed here. It should be noted that freezing the extract solid in the deep-freeze results in rapid loss of activity.

Tubes for Blood Specimens.—Citrate is used as the anticoagulant rather than oxalate because of the possibility of loss of prothrombin by adsorption on the precipitate of calcium oxalate. However, the possibility of deterioration of citrate solutions as a result of the growth of moulds or bacteria has apparently received little attention. The use of contaminated tubes may result in grossly misleading values. Autoclaving the stoppered tubes containing citrate is more likely to ensure sterility than simply pipetting a sterile citrate solution into the tubes under aseptic conditions.

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A CRITICAL EVALUATION OF RED CELL AND PLASMA VOLUME TECHNIQUES IN PATIENTS WITH BURNS

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From the results in 110 patients, in whom 207 red cell volume estimations were made at various times after burning using either ^{51}Cr-, ^{32}P-, or Group NN-labelled cells, and 17 plasma volume estimations on the same patients using either T-1824- or ^{131}I-labelled albumin, it is concluded that:

(*a*) The ^{51}Cr red cell volume method is the most reliable method; it gives results which are probably not in error by more than $\pm 10\%$ when the labelled cells are washed three times and so has a place in the assessment of the changes in red cell volume that follow a severe burn. In conjunction with a well type scintillation counter the radiation dosage received by the patient with ^{51}Cr is about one-fifth of that with any other method.

(*b*) The ^{32}P method is slightly less reliable, as shown by on average higher supernatant radioactivity levels and a greater scatter in the rate of disappearance of the isotope from the circulation.

(*c*) The Ashby (Group NN) method and the plasma volume and haematocrit method are inaccurate in patients with burns.

The changes in circulatory volume that follow a severe burn may be divided into those affecting plasma and those affecting red cells. In moderately severe burns up to about one-third of body surface, any loss of red cells, if it occurs at all, is so small as to be undetectable by the methods currently available, and a change in plasma volume is reflected by a change in the haematocrit. With large burns, however, replacement of the lost plasma by a suitable colloid using the haematocrit as a guide to the level of the plasma volume may lead to oligaemia owing to an unknown loss of red cells. It is in these cases that methods of estimating red cell volume may be of value.

The accuracy of a red cell volume estimation is mainly dependent upon complete mixing of the injected labelled cells in the circulation before sampling. However, in patients with severe burns the peculiar circulatory state with gross leakage of plasma and venoconstriction might be expected to impair mixing and hence the accuracy of blood volume estimations. An investigation was therefore carried out to show whether red cell volume estimations were likely to be accurate enough to improve the clinical management of patients with severe burns. The methods were similar to those previously described for patients with injuries other than burns (Davies and Topley, 1959).

METHODS

Two hundred and seven estimations of red cell volume using ^{32}P- or ^{51}Cr-labelled red cells, and 17 plasma volumes using T-1824- or ^{131}I-labelled human albumin, have been made on 110 patients with burns involving between 10 and 90% of the body surface. About half the patients were children under 12 years old. Most of the estimations (167/224) were made within 48 hours of burning.

Red Cells Labelled with Radioactive Phosphorus [^{32}P]

The technique based on that of Chaplin (1954) has already been described in detail (Davies and Topley, 1959).

Red Cells Labelled with Radioactive Chromium [^{51}Cr]

The following slight modifications of the technique described by Davies and Topley (1959) are based on the method of Mollison and Veall (1955). The dosage of radioactivity injected into the children did not exceed 0.5 μC/kg. body weight with burns of less than 45% of the body surface and with larger burns it did not exceed 2 μC/kg. During the last six months of the five-year investigation a well type scintillation counter superseded the annular type. The increase in sensitivity of the equipment for the detection of the isotopes emitting γ rays (^{51}Cr and ^{131}I) was approximately seven times, i.e., for ^{51}Cr, 1 μC changes from 9.530 counts per minute to 65.660 counts per minute. This change made possible a fivefold reduction in the

acetone in a mortar. (The cerebellum is best excluded on account of the difficulty of removing completely the membranes and blood vessels.) The supernatant fluid is decanted and replaced with fresh acetone and the maceration continued, this procedure being repeated until a friable mass is obtained. The tissue is sufficiently dehydrated when a small portion, removed from the mortar and allowed to dry, can be crumbled to a dry powder. The acetone is then decanted and the brain is spread on large sheets of filter paper to absorb the excess of solvent and allowed to dry. Finally the dried brain, after thorough mixing, is placed in jars and stored in vacuum desiccators containing phosphorus pentoxide and kept in the refrigerator. (The main stock of brain is stored in one desiccator and a smaller amount in another from which it is weighed out as required. In this way the main stock of material is not exposed unnecessarily to air, moisture, and changes in temperature.)

Preparation of Thromboplastin Emulsion.—Dried brain, 2 g., is extracted at 37° C. for 15 minutes with 20 ml. of 0.85% w/v aqueous sodium chloride containing 0.5% w/v phenol, and stirred at five-minute intervals. After a few hours' standing to allow visible particles to settle, the supernatant fluid is removed ("stock thromboplastin extract"). This should be stored in the refrigerator but not frozen solid. The optimum concentration of thromboplastin for routine use is determined as follows. Normal blood is collected in a citrated tube (see below). The plasma is separated and diluted with four volumes of 0.85% w/v sodium chloride. Then 0.1 ml. of the diluted plasma is mixed with 0.1 ml. of stock thromboplastin extract, the mixture is incubated at 37° C. for 30 seconds, 0.1 ml. of 0.02M calcium chloride is blown in, and the time taken for clotting is noted. The test is repeated with dilutions of stock thromboplastin extract in phenol-saline (e.g., 1+1, 1+2, and so on), and the dilution giving the shortest clotting time is selected ("diluted thromboplastin extract"). A portion or all of this diluted thromboplastin extract is then mixed with an equal volume of 0.02M calcium chloride solution for routine use ("thromboplastin-calcium reagent"). This mixture, stored at 4° C. to 8° C., retains its potency for several weeks (Fig. 1).

Preparation of Citrated Tubes.—Amounts, each of 0.2 ml., of a 3.8% w/v aqueous solution of sodium citrate dihydrate (stored in the refrigerator) are measured into tubes (75 × 10 mm.) marked at exactly 2 ml. The tubes are then securely stoppered with rubber bungs and are autoclaved at 15 lb. for 20 minutes. It is convenient to autoclave the tubes in stainless steel racks stacked on top of one another and held together by rods, the top layer of tubes being covered by a metal plate: in this way the bungs are kept in place and no air enters when the tubes are allowed to cool. After autoclaving, each tube is labelled with a "sterile" label bearing the date of preparation and covering the bung. Tubes are issued to the wards and out-patient department with instructions that they be stored in an upright position.

Collection of Blood.—Blood is collected in the usual way and the tube is filled exactly to the 2 ml. mark. The plasma is separated by centrifugation.

The Test.—Thromboplastin-calcium reagent, 0.2 ml., is placed in a small test-tube (about 60 × 10 mm.), which is then left in the water-bath at 37° C. for 20 to 30 seconds. Citrated plasma at room temperature, 0.1 ml., is blown in, a stop-watch being started at the same time. The tube is slanted, and rotated gently about its long axis until the first appearance of fibrin strands. These are seen clearly if the tube is held underneath a light and against a dark background. The test is performed in duplicate at least. (When the test is repeated the tube is kept in the water-bath to within about five seconds of the time indicated by the first estimation.) Normal plasma specimens are tested in the same way and the result obtained with the patient's plasma is expressed as a ratio, viz.:

$$\text{Prothrombin clotting time ratio} = \frac{\text{Clotting time observed with patient's plasma}}{\text{Average clotting time observed with normal plasma}}$$

Notes on the Method

Preparation and Storage of Dried Brain.—These are essentially as described by Aggeler, Howard, Lucia, Clark, and Astaff (1946), with slight modifications. In our experience the dried brain, stored as described, retains its potency for at least a year.

Standardization of Thromboplastin.—The method is that of Biggs (1951), except for the use of phenol-saline.

Thromboplastin Extract.—The use of phenol to preserve emulsions of fresh brain was suggested by Toohey (1950); and Powell (1957), working in this laboratory, showed that extracts of dried brain can be preserved in the same way for at least two weeks. Powell's findings can be confirmed here. It should be noted that freezing the extract solid in the deep-freeze results in rapid loss of activity.

Tubes for Blood Specimens.—Citrate is used as the anticoagulant rather than oxalate because of the possibility of loss of prothrombin by adsorption on the precipitate of calcium oxalate. However, the possibility of deterioration of citrate solutions as a result of the growth of moulds or bacteria has apparently received little attention. The use of contaminated tubes may result in grossly misleading values. Autoclaving the stoppered tubes containing citrate is more likely to ensure sterility than simply pipetting a sterile citrate solution into the tubes under aseptic conditions.

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A CRITICAL EVALUATION OF RED CELL AND PLASMA VOLUME TECHNIQUES IN PATIENTS WITH BURNS

BY

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From the Medical Research Council Industrial Injuries and Burns Research Unit, Accident Hospital, Birmingham

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From the results in 110 patients, in whom 207 red cell volume estimations were made at various times after burning using either ^{51}Cr-, ^{32}P-, or Group NN-labelled cells, and 17 plasma volume estimations on the same patients using either T-1824- or ^{131}I-labelled albumin, it is concluded that:

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The changes in circulatory volume that follow a severe burn may be divided into those affecting plasma and those affecting red cells. In moderately severe burns up to about one-third of body surface, any loss of red cells, if it occurs at all, is so small as to be undetectable by the methods currently available, and a change in plasma volume is reflected by a change in the haematocrit. With large burns, however, replacement of the lost plasma by a suitable colloid using the haematocrit as a guide to the level of the plasma volume may lead to oligaemia owing to an unknown loss of red cells. It is in these cases that methods of estimating red cell volume may be of value.

The accuracy of a red cell volume estimation is mainly dependent upon complete mixing of the injected labelled cells in the circulation before sampling. However, in patients with severe burns the peculiar circulatory state with gross leakage of plasma and venoconstriction might be expected to impair mixing and hence the accuracy of blood volume estimations. An investigation was therefore carried out to show whether red cell volume estimations were likely to be accurate enough to improve the clinical management of patients with severe burns. The methods were similar to those previously described for patients with injuries other than burns (Davies and Topley, 1959).

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amount of ^{51}Cr required for a red cell volume estimation and thus also in the radiation dosage received by the patient.

As occasionally it was not possible to obtain venous blood for the sample taken after mixing was assumed to be complete, 0.5–1.0 ml. of capillary blood from a free-flowing ear or toe prick was used.

Red Cells Labelled with the Group NN Antigen

The method as described by Mollison (1951) was used.

Plasma Proteins Labelled with T-1824

The method described by Davies and Topley (1959) was used on 12 occasions in conjunction with the ^{32}P red cell volume method.

Human Albumin Labelled with Radioactive Iodine [^{131}I]

The published method (Davies and Topley, 1959) was used on five occasions in conjunction with the ^{51}Cr red cell volume method.

Other Methods and Calculations

The blood volume was calculated from either the plasma volume or the red cell volume and the venous and body haematocrits. The ratio between the body and venous haematocrits was taken as 0.90 (Davies and Topley, 1959).

RESULTS

The factors which may cause inaccuracy will first be described. In the second section will be given results of simultaneous estimations using various pairs of methods, and the third section will show the consistency between sequential estimations before and after an operation at which there was a known blood loss and transfusion.

Factors which May Cause Inaccuracy

Radioactivity in the Supernatant.—The radioactivity in the solution in which the labelled cells were suspended (the supernatant) has been expressed as a percentage of the total quantity of radioactivity injected (Table I). The results obtained during the first 48 hours after burning have been compared with those obtained later. A comparison has also been made between different isotopes and the number of washes with saline alone or plasma saline. There were significantly more results above 1% when ^{32}P was used (27 out of 97 or 28%) than when ^{51}Cr was used (seven out of 92 or 8%) (σ=0.689, t=3.94, P=<0.001). The data also suggest that three washes of ^{51}Cr-labelled cells are better than two for reducing the number of observations above 1%.

TABLE I

RADIOACTIVITY LEVEL OF PLASMA SALINE (SUPERNATANT) IN WHICH THE LABELLED CELLS WERE SUSPENDED AS A PERCENTAGE OF RADIOACTIVITY INJECTED

No. of Washes	Isotope	Number of Observations	Mean Percentage in Supernatant	Number of Observations Over 1%	Observations Over 1% as % Total Observations
Patients' cells labelled during first 48 hours after burning					
3	^{32}P	52	0·72	13	25
3	^{51}Cr	17	0·29	0	0
2	^{51}Cr	66	0·46	6	9
Patients' cells labelled after first 48 hours after burning					
3	^{32}P	45	0·95	14	31
3	^{51}Cr	1	0·53	0	0
2	^{51}Cr	8	0·51	1	12½
Cells washed with plasma saline					
3	^{32}P	34	0·56	7	20
3	^{51}Cr	3	0·27	0	0
2	^{51}Cr	29	0·48	3	10
Cells washed with saline alone					
3	^{32}P	27	0·92	10	37
3	^{51}Cr	2	0·29	0	0
2	^{51}Cr	35	0·47	3	9

It has also been shown that washing ^{32}P-labelled cells with plasma saline compared with saline alone results in a small number of observations above 1%.

Adequacy of Intravascular Mixing and Survival of Labelled Materials in the Circulation.—The changing levels of radioactivity in the circulation have been plotted for each patient, when multiple venous sampling was possible as the concentration of label per unit volume of red cells (log scale) against the time after injection (linear scale). Extrapolation of the best straight line through these points to zero time gives the theoretical initial concentration (100%), and other observations are expressed as a percentage of this value. The mean of all the percentages at each time interval after injection together with the standard deviations are plotted in Figs. 1a and 1b for each type of radioactive label. Most of the observations were made within 48 hours of burning. Thirty estimations on 25 patients have been made with ^{51}Cr and 22 estimations on 15 patients with ^{32}P. The best straight line has been drawn through the mean values to indicate the average rate of disappearance. Ten observations were made with T-1824. With the Ashby (Group NN) technique only one blood sample was taken at the end of the transfusion so it was not possible to show whether mixing had been adequate.

On 12 occasions venous sampling at approximately two-hour intervals was possible for up to 18 hours after the injection of ^{51}Cr-labelled red cells. The data (Fig. 1c) have been calculated by the same means as the observations made within

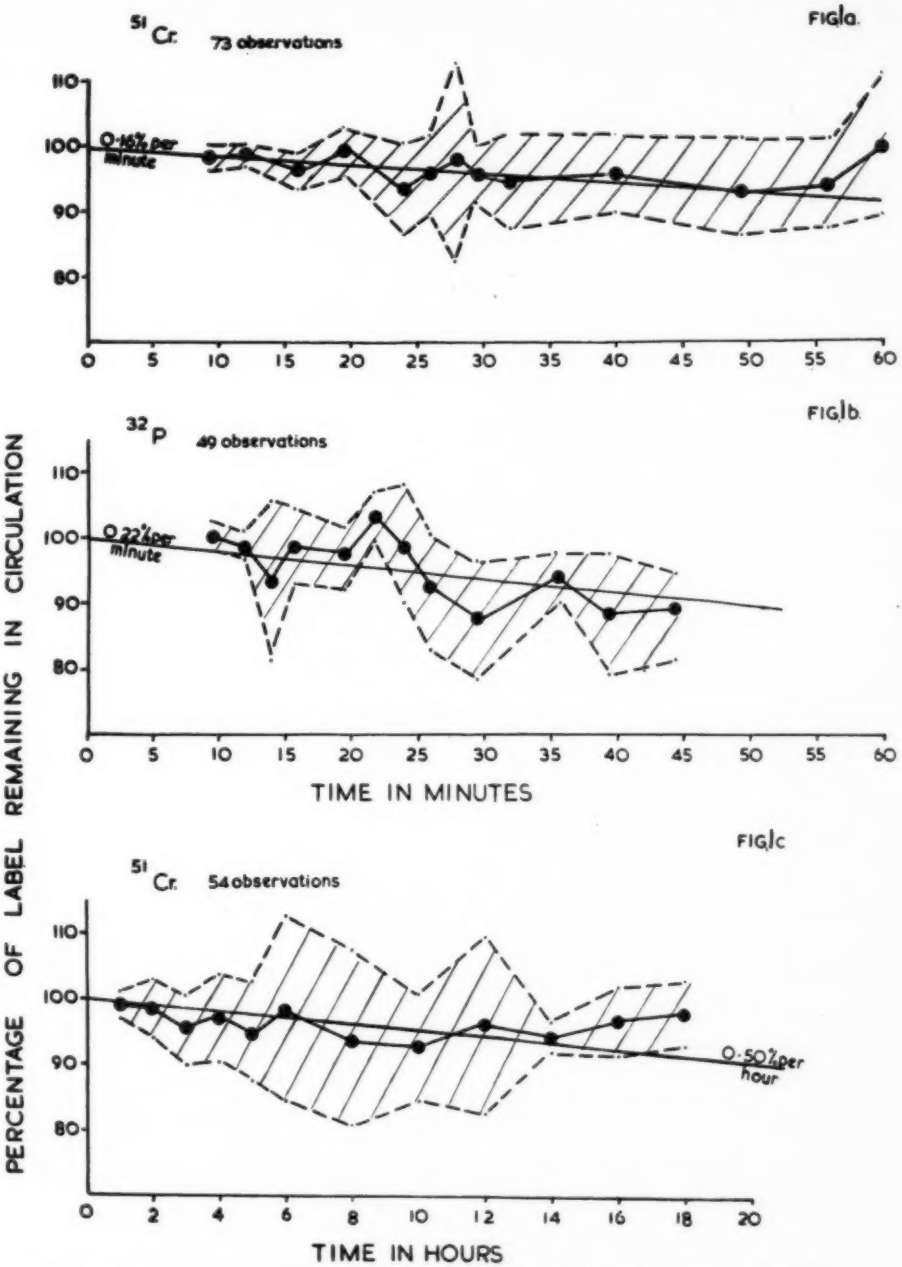
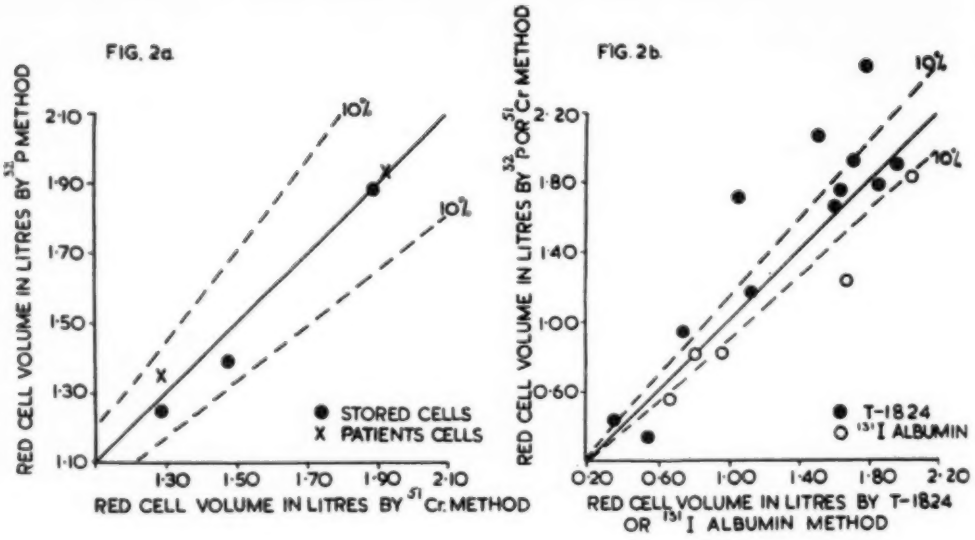


FIG. 1.—The mean rates of disappearance of ^{51}Cr- and ^{32}P-labelled red cells. The shaded area shows the standard error.



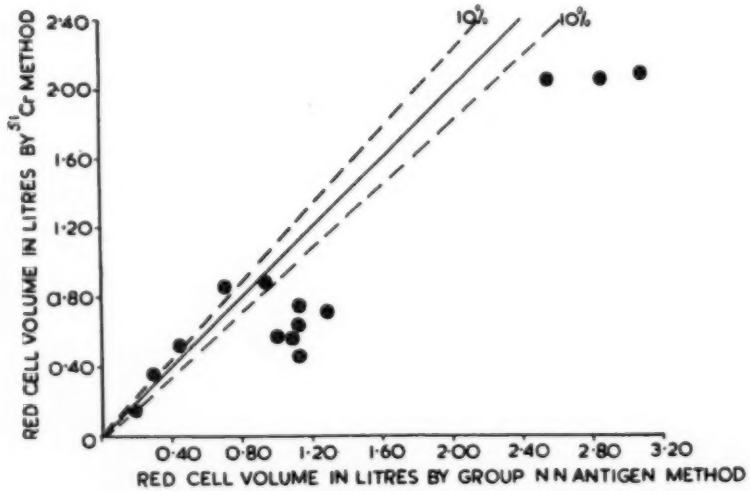


FIG. 2.—Simultaneous estimations of red cell and plasma volume using a number of methods. The scatter around the line of equivalence is shown together with the ±10% limits.

one hour of injection. The 10 patients had burns ranging between 18 and 65% of the body surface.

It can be seen (Table II) that with ^{51}Cr none of the 34 results taken between nine and 20 minutes after injection varied from the mean value by more than $\pm 10\%$. With ^{32}P, two out of 29 exceeded these limits during the same period. The

TABLE II

THE NUMBER OF OBSERVATIONS EXCEEDING $\pm 10\%$ OF MEAN VALUES ILLUSTRATED IN FIG. 1

Labelling Agent	9–14 Min.	15–20 Min.	21–30 Min.	31–40 Min.	Total
^{51}Cr	0/11	0/23	2/18	1/10	3/62
^{32}P	1/9	1/20	1/11	2/7	5/47

number of observations outside $\pm 10\%$ of the mean increased as the time between injection and sampling increased. The observations over 18 hours after injection show a greater scatter than those over one hour. Nevertheless only three of the 54 observations exceeded $\pm 15\%$ of the mean values.

Variations in Ratio between Body and Venous Haematocrits.—Seventeen simultaneous estimations of red cell and plasma volume using ^{32}P or ^{51}Cr- and T-1824- or ^{131}I-labelled albumin enabled the body haematocrit to be calculated and hence its ratio to the venous haematocrit. Eight of the 17 observations were made within 48 hours of burning. The mean ratio was 0.931. Seven of the observations lay outside the limits of $\pm 10\%$, whilst only two lay outside $\pm 20\%$ of the mean value.

Simultaneous Estimation of Red Cell and Plasma Volume.—These estimations were made by two methods in parallel on the same subjects. The labelled materials were injected from two different syringes with not more than one-minute intervals between the injections. The amount of the two labels was determined separately in the sample of blood taken after mixing was complete. The equivalence of the results is shown in Fig. 2a, b, c.

Red Cells Labelled with Both ^{32}P and ^{51}Cr.—The five pairs of estimations using both ^{32}P and ^{51}Cr (Fig. 2a) agree within a range of $\pm 7\%$ of the line of equivalence. For three of the five experiments red cells stored in A.C.D. for transfusion purposes were labelled with ^{51}Cr whilst the patients' own cells were labelled with ^{32}P. In the remaining two experiments the patients' own cells were labelled with both isotopes.

Red Cells Labelled with ^{51}Cr and the Group NN Antigen.—The 14 simultaneous estimations show a considerable scatter (Fig. 2c). Nine of the estimations lie outside $\pm 20\%$ of the line of complete equivalence. On average the red cell volume calculated from the Group NN antigen method was higher than that estimated by ^{51}Cr.

Simultaneous Estimations of Red Cell and Plasma Volume Using ^{32}P- and ^{51}Cr-labelled Red Cells and T-1824- or ^{131}I-labelled Albumin.—The ^{32}P-labelled red cells and T-1824 were injected within one minute of each other, whereas the ^{51}Cr-labelled red cells and ^{131}I-labelled albumin were usually injected with 15 to 20 minutes between them so that the red cell volume estimation was not affected by the plasma volume radioactivity.

Twelve simultaneous estimations were made using ^{32}P and T-1824 (solid circles, Fig. 2b), and five using ^{51}Cr- and ^{131}I-labelled albumin (open circles, Fig. 2b). The scatter of the result is considerably greater than that observed with simultaneous red cell volume estimations, only seven of the 17 lying within $\pm 10\%$ of the line of complete equivalence. Eight of the 17 estimations were made within 48 hours of burning.

Sequential Estimations Before and After a Known Blood Loss and Transfusion

On 12 occasions a red cell volume estimation using either ^{32}P or ^{51}Cr was made before and another after a primary excision and grafting operation at which there was a measured blood loss by swab weighing and a known volume of blood transfused. The results (Table III) have been calculated by first converting volumes of blood lost and transfused into volumes of red cells lost and transfused using the haematocrit, and then comparing the difference between the volume of red cells lost and transfused with the

TABLE III

DIFFERENCE BETWEEN KNOWN BLOOD LOSS AND TRANSFUSION AND TWO RED CELL VOLUME ESTIMATIONS

Estimated Area of Burn (%)	% of Body Surface Excised	Error as % of Normal Red Cell Volume
13	8	+10·9
20	9	+0·6
13	11	+1·9
25	13	−8·5
32	15	−7·8
33	15	−3·2
24	15	+5·6
18	15	+26·7
38	15	−19·2
25	18	+5·3
30	21	−15·3
30	22	−16·1

The difference is shown as a percentage of the patient's normal red cell volume. The estimated area of the burn and the area excised are also shown.

difference between the pre- and post-operation red cell volume estimations. These observed differences have been expressed as a percentage of the patients' expected red cell volume for height. It can be seen that with burns up to 38% of the body surface, and when up to 22% of the body surface was excised, the error between expected and found values only exceeded ±20% once and only on five occasions did it exceed ±10%. Larger excisions on larger burns were excluded because of obvious and unmeasured post-operative haemorrhage.

DISCUSSION

This study has revealed variations in the amount of radioactivity in the suspending saline, variations in the mean rate of disappearance of the labelled materials from the circulation, and differences between two methods in parallel. These variations will be compared with findings in patients with other injuries, and discussed in terms of their probable effect on the accuracy of red cell volume measurement after burns.

Factors Causing Variation

Level of Radioactivity in the Supernatant.—This indicates that red cells from patients with burns are less satisfactorily labelled with ^{32}P compared with red cells from patients with injuries as there were two to three times as many observations above 1% (Davies and Topley, 1959). The data suggest that ^{51}Cr-labelled red cells washed three times is the preferred method even in the absence of plasma saline. This is in contrast to the evidence in normal individuals (Mollison, 1958), where only two or sometimes one wash is necessary.

The Adequacy of Intravascular Mixing and the Survival of Labelled Materials in the Circulation.—The concentration of the labelled material per unit volume of blood at various times up to 40 minutes after injection was not significantly different from that found in normal individuals or for patients with injuries other than burns (Davies and Topley, 1959). These data, together with the observations up to 18 hours after injection, suggest that mixing was complete by nine to 20 minutes after injection and that no preferential disappearance of labelled cells occurred in this time. A single sample during this time is therefore probably a fair sample for a red cell volume estimation. The chances of a single observation exceeding ±10% of the mean value are shown in Table II.

The Ratio Between the Body and Venous Haematocrits.—Patients with burns showed a range of ratios similar to those found in patients with other injuries (Davies and Topley, 1959), and thus, as in normal individuals, errors of ±10–20% could at times be produced in a red cell volume when calculated from a plasma volume and the haematocrit.

Simultaneous Estimations by Different Methods

The simultaneous red cell volume estimations using ^{32}P and ^{51}Cr show good agreement similar to that found in patients with injuries other than burns and in normal individuals (Davies and Topley, 1959) whereas the Ashby technique using Group NN cells has often proved inaccurate.

The simultaneous red cell and plasma volume estimations have shown a greater scatter than that observed in patients with injuries other than burns. About one-third of the results (in six out of 17 patients) exceeded ±20% of the line of equivalence compared with only one-sixth in patients with injuries other than burns (Davies and Topley, 1959). The results suggest that the plasma volume and haematocrit method is not a suitable way of estimating red cell volume in patients with burns, probably because of the changes in capillary permeability which may lead to a leakage of the dye or labelled albumin from the circulation.

Radiation Dosage

The considerations of radiation dosage due to ^{51}Cr, ^{32}P, and ^{131}I already described (Davies and Topley, 1959) also apply to patients with burns. In general, however, smaller doses of the isotopes were used, as in the children the dosage of ^{51}Cr never exceeded 0.5 μC/kg. with burns of less than 45% of the body surface and 2.0 μC/kg. when the burned area exceeded this value.

In adult patients 25 μC was usually given and gave a radiation dosage of 0.025 r.e.p. on the assumption that the isotope was evenly distributed throughout the body of a 70 kg. patient. A similar radiation dosage was received from the 2 μC of ^{32}P required for a red cell volume estimation using this isotope. The reduction in the amount of ^{51}Cr required from 25 μC to 5 μC by use of the well type scintillation counter reduced the radiation dosage to 0.005 r.e.p. in the more recently burned patients.

I am grateful for the help given by Dr. Mary Fisher, Mrs. Ann Foster, Miss Janet Frost, and Miss Toni Boulter in the red cell volume estimations, and by Mrs. Myra Evans in the T-1824 estimations.

I wish to acknowledge the helpful advice and criticism given by Dr. E. Topley and Dr. J. P. Bull

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J. clin. Path. (1960), 13, 112.

THE DEVELOPMENT OF AN ACQUIRED HAEMOGLOBIN ABNORMALITY CAUSED BY THERMAL INJURY

BY

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From the Medical Research Council Industrial Injuries and Burns Research Unit, Birmingham Accident Hospital

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Alkali denaturation of the haemoglobin derived from burned patients at various intervals from the time of the injury was investigated. A delayed denaturation curve was always found.

The involvement of the transfused cells was established by a differential agglutination technique.

Red cell populations were fractionated into two portions, one of which was probably enriched in younger cells. This portion was preferentially affected.

A starch gel technique yielded a " slow " and a " fast " fraction of haemoglobin with differing denaturation rates, even in normals. The " slow " fraction appeared to be increased quantitatively and qualitatively, if compared with normal blood.

In 1951, Singer, Chernoff, and Singer had found that abnormal haemoglobins other than foetal haemoglobins showed a diminished alkali denaturation rate. Künzer (1955) described the occurrence of increased amounts of alkali-resistant haemoglobin, which he termed " foetal," in 47 children, all above 5 years of age and suffering from anaemia of varying aetiology. No quantitative relationship between the alkali-resistant fraction and the severity of the anaemia was found. Since these papers were published much chemical work has been done on the amino-acid composition of haemoglobin F, which was found to possess only 2.2 valine residues as compared with 4.6 in haemoglobin A (Huisman and Drinkwaard, 1955). The work of Perosa and Bini in 1954, however, showed that alkali-resistant haemoglobin was not necessarily identical with haemoglobin F.

The present study was undertaken in an effort to demonstrate a possible indirect thermal damage to the haemoglobin of burned patients.

Materials and Methods

Patients.—Sixteen patients were studied in detail; their ages ranged from 2 years to 70 years. Five of these were adults. The surface areas involved ranged from 16 to 90%.

Methods.—Heparinized blood was collected from the patients and immediately washed three times with 1% NaCl and the packed cells lysed by the addition of approximately 1 vol. of distilled water, followed by freezing and thawing. The stromata were removed by 10 minutes' centrifugation in an angle centrifuge at 5,000 r.p.m. All specimens were stored at −15° C. before use and were not kept longer than one week before testing.

The haemoglobin solutions were examined for their rates of denaturation by dilute alkali. Oxygen was always bubbled through before testing. The rate of denaturation was studied by a suitable modification of the method described by Brinkman and Jonxis (1935). The haemoglobin solution was diluted to 0.15–0.3 g.% with 0.04% ammonia solution and 2.4 ml. of this solution was treated with 0.1 ml. N NaOH. The *p*H of this solution was 12.5.

The increase in optical density was noted at 10-second intervals in a Hilger " biochem " absorptiometer, which had been modified as follows. The galvanometer was replaced by a Cambridge spot galvanometer which was damped by a total resistance of 20k. A 5k resistor was placed across the photocell output. A portion of a " unicam " S.P.500 cell carriage was mounted in the light path and two precisely matched 10 mm. depth cells were used for the measurements. A Wratten 25 filter, which had 12.6% transmission at 590 mμ and 87.6% at 650 mμ, mounted between optical glass was used. Denaturation was assumed to be complete after 12 hours' incubation at 37° C. The method described made it possible to obtain accurate readings towards the completion of denaturation and yet utilize the high extinction coefficient of alkali haemochromogen at that wavelength as compared with 576 mμ. Fig. 1 shows the normal curve obtained using the blood of 12 young normal adults.

Some fractionation experiments were also carried out in order to obtain either a fraction enriched in old cells or young cells or transfused cells, the last having been exposed to the patient's circulation.

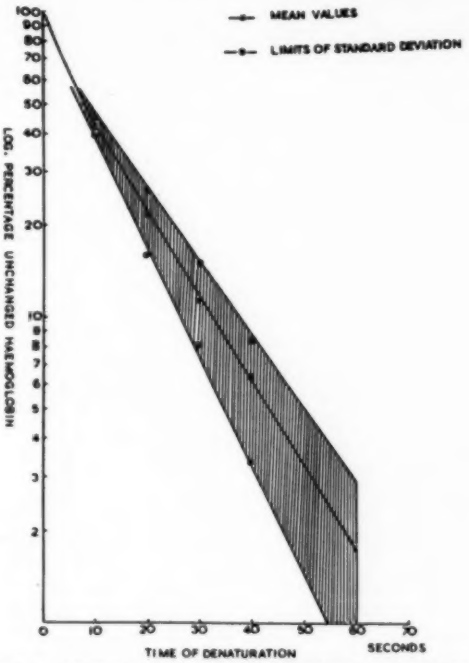

FIG. 1.—Alkali denaturation curve of normal blood (12 individuals).

Washed red cells from a burned patient were exposed to 0.4 g.% NaCl at 25° C. for 60 minutes. This yieded two fractions. The supernatant, somewhat enriched in older cells, was dialysed and concentrated by ultrafiltration. The other fraction, which was somewhat enriched in younger cells, was treated as usual. A similar scheme of age group fractionation has been described by Simon and Topper (1957). Gravitational fractionation was attempted on another sample. Thirty per cent. albumin was diluted to 20% with physiological saline. A Wintrobe tube was used for fractionation. This was filled to the 2 cm. mark with an approximately 10% cell suspension and to the 6 cm. mark with 20% albumin. The contents were then mixed gently with a fine pipette and the top 3 cm. layer was separated after standing for one hour at room temperature. This yielded a top fraction enriched in younger cells and a bottom fraction enriched in older cells. Several tubes were put up simultaneously and the appropriate fractions pooled.

The haemoglobin of transfused cells and the patient's own cells was obtained by the transfusion of M-negative blood and fractionating a sample obtained after a suitable time interval with anti-M serum. Films of the supernatant cells (transfused cells) showed less than 1% of the cells agglutinated after further treatment with anti-M serum.*

*This fractionation was kindly carried out by Dr. E. Topley.

Some haemoglobin solutions were fractionated by means of a starch gel electrophoretic technique as described by Baar (1958) using 0.05 M "tris" buffer as the electrolyte. After two hours' electrophoresis, separation into three zones could be observed. These, however, were not sharply demarcated. The complete width of the three zones was therefore divided into equal halves and the region nearest the cathode was termed "slow." Fig. 2 shows the curves obtained from normal blood by alkali denaturation of the isolated zone material.

Results

The patients studied were divided into four groups according to the area of the burn. The 30-second "alkali-resistant" fraction was chosen to represent graphically the quantitative variations of this fraction in relation to the time of the injury, the area involved in the burn, and the timing and the extent of the blood transfusion. Table I shows the ages, the body areas burned, the presence or absence of haemoglobinuria, and details of the primary excisions of the cases investigated. The denaturation curves of these cases are shown in

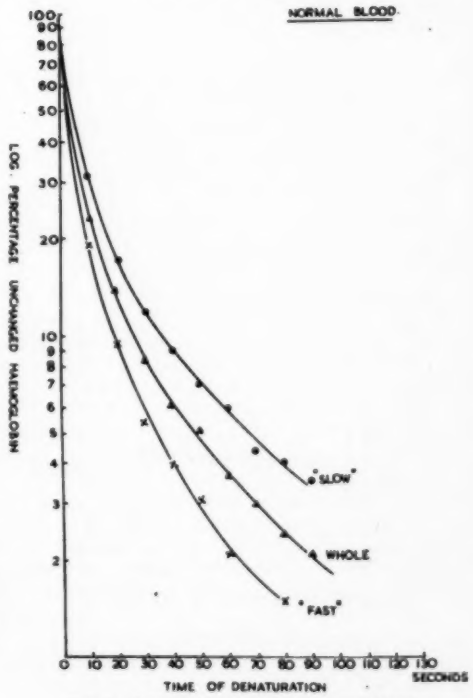

FIG. 2.—Starch gel fractionation of normal blood.

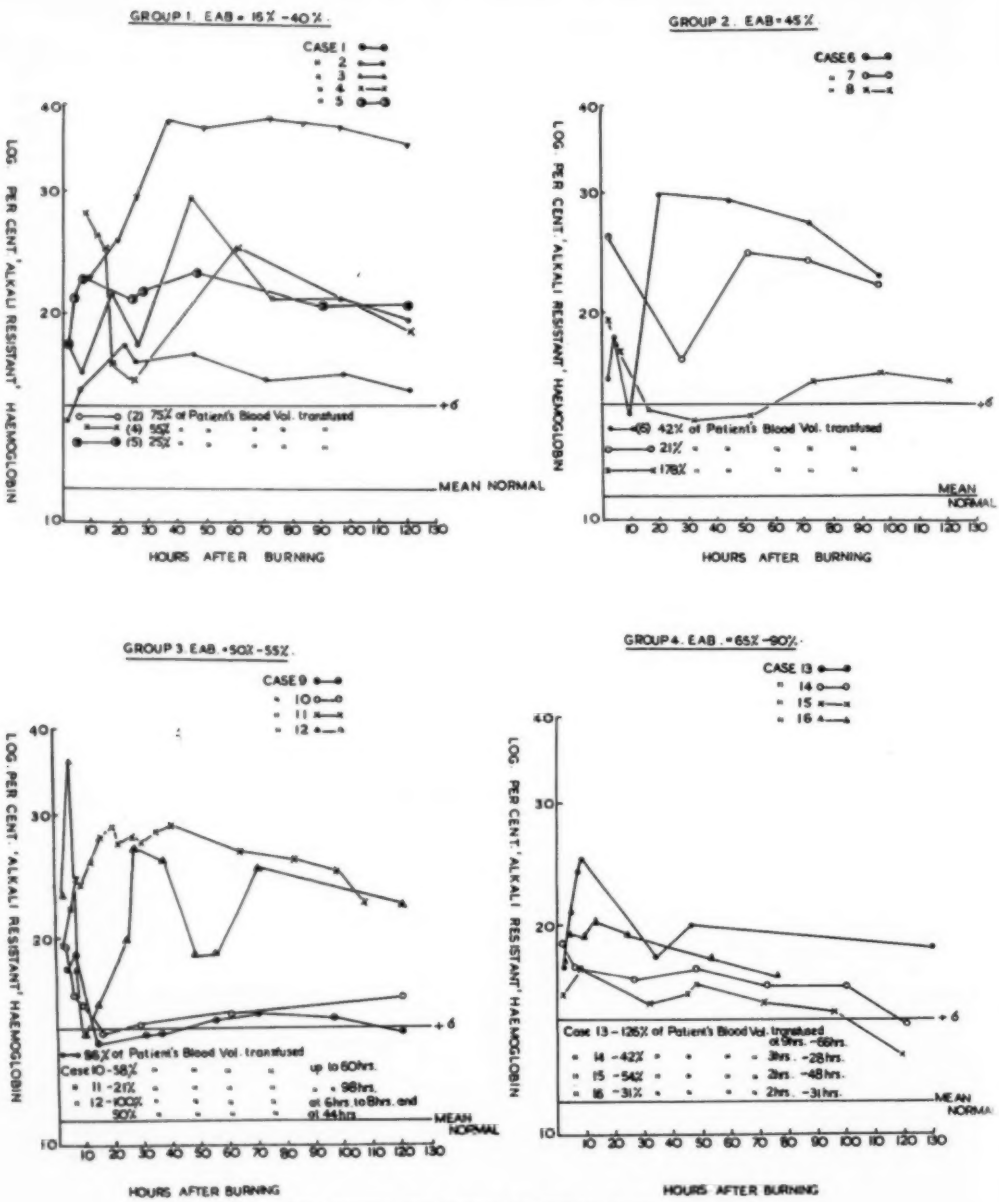
GROUP 1. EAB = 16% - 40%.
CASE 1
= 2
= 3
= 4
= 5
LOG PER CENT 'ALKALI RESISTANT' HAEMOGLOBIN
(2) 75% of Patient's Blood Vol. transfused
(4) 55% = = = = =
(5) 25% = = = = =
+6
MEAN NORMAL
HOURS AFTER BURNING
GROUP 2. EAB = 45%
CASE 6
= 7
= 8
(6) 42% of Patient's Blood Vol. transfused
21% = = = = =
178% = = = = =
MEAN NORMAL
GROUP 3. EAB. = 50% - 55%.
CASE 9
= 10
= 11
= 12
96% of Patient's Blood Vol. transfused
Case 10 - 58% = = = = up to 60hrs.
= 11 - 21% = = = = = 98hrs.
= 12 - 100% = = = = at 6hrs. to 8hrs. and
90% = = = = at 44 hrs.
GROUP 4. EAB. = 65% - 90%.
CASE 13
= 14
= 15
= 16
Case 13 - 126% of Patient's Blood Vol. transfused at 9hrs. - 66hrs.
= 14 - 42% = = = 3hrs. - 28 hrs.
= 15 - 54% = = = 2hrs. - 48 hrs.
= 16 - 31% = = = 2hrs. - 31 hrs.

FIG. 3.—The 30-second alkali-resistant haemoglobin fraction of Cases 1–16.

TABLE I

Case No.	Age (yr.)	Estimated Area of Body Surface Burned (%)	Estimated Area of Full-thickness Skin Loss (%)	Haemoglobinuria	Primary Excision
1	11	16	13	0	0
2	2	35	10	L*	0
3	70	35	30	E*	0
4	1½	37	35	E	0
5	65	40	35	0	0
6	9	45	17	L (red cells)	0
7	3	45	40	0	0
8	7	45	20	E	+
9	8½	50	20	E, L (red cells)	+
10	4	50	45	E, L	+
11	36	55	50	0	0
12	8	55	50	—	+
13	20	65	35	0	+
14	45	80	30	L	0
15	10	85	80	E, L	0
16	5	90	90	E, L	0

* E = early (0–12 hours) and L = late (at least three haemoglobin-stained urines 12–24 hours).

Fig. 3. In all groups there is an increase in the alkali-resistant fraction and this abnormality is maintained for some time. Cases 6, 7, and 12 show secondary rises, which develop after the transfusion. In Group 1, Cases 1 and 3 did not receive any transfusion. The former only shows a minor development of the more resistant fraction, the maximum being reached within 24 hours and a return to normal levels within one week. Case 3, an adult, shows this maximum level about two days after injury. This is associated with a much slower return to normal. The other adult cases studied, namely, 5, 11, 13, and 14, do not show this delay. This fact may be due to the timing of the transfusion. Case 2 showed a severe haemolytic reaction with haemoglobinaemia and haemoglobinuria, both the transfused and the patient's blood being involved. Yet the level of the abnormal haemoglobin rose in spite of the dilution of the patient's blood by transfused cells and the increased rate of red cell destruction. Such a finding is highly suggestive for the abnormality developing not only in the patient's blood but also in the transfused cells. Case 4, a patient who was transfused after the initial blood sampling, shows that approximately 15% of the patient's cells may be involved in as little as eight hours after the injury. Case 7 in Group 2 and Cases 10 and 12 in Group 3, whose samples were obtained before transfusions, all confirm the relative rapidity of the change. Case 11, whose transfusion was only started after the third sampling, clearly demonstrates a qualitative increase in this abnormality. This could be due either to a larger number of cells becoming involved or to the already affected haemoglobin increasing in abnormality.

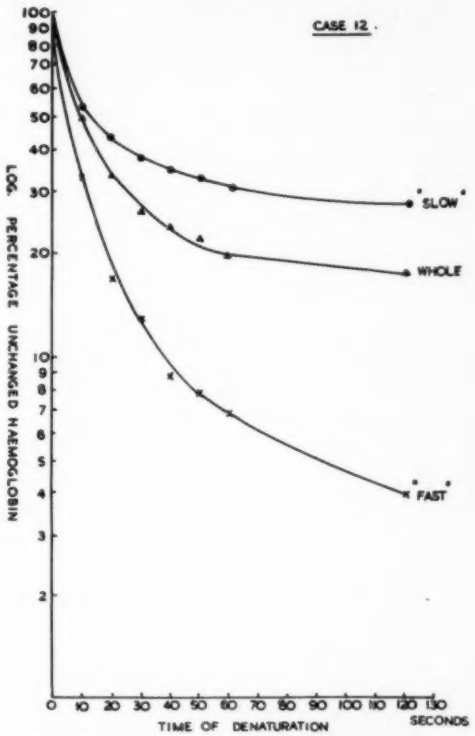

FIG. 4.—Starch gel fractionation of the blood of Case 12.

Starch gel fractionation on normal blood has shown that the slight tailing of the denaturation curve of normal blood (Baar and Hickmans, 1941) was due to a component of slower denaturation than the main portion, which also migrated more slowly on starch gel. This is shown in Fig. 2. Starch gel fractionation experiments on Case 12 are shown in Fig. 4. Two fractions were obtained, both with a delayed denaturation curve as compared with the normal. The "fast" fraction from the patient approximates the "slow" fraction from normal blood. Although both fractions appear to be affected to some extent, the "slow" fraction's denaturation is delayed about twice as much as the "fast" fraction. This delay in the denaturation of the "fast" fraction could reasonably be due to the incomplete method of separation.

Scrutiny of the curves obtained from the patient's blood in relation to the extent and timing of the transfusion reveals that transfusions involving about one blood volume or more

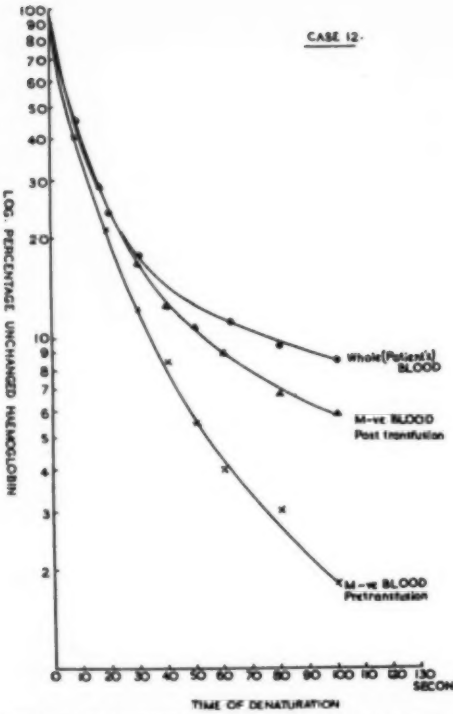

FIG. 5.—Alkali denaturation of whole (patient's) blood and transfused blood.

abolish the abnormality at least temporarily. The abnormality, however, reappears, except in Cases 9 and 10. The first of these patients received a very extensive blood transfusion lasting up to 10 hours after the injury and the second case was moderately and slowly transfused. The failure of the abnormality to reappear again in these two cases may be due to dilution of a plasma factor acting on the red cells—in Case 9 to a very low level and in Case 10 for a long period to a low level. Experiments *in vitro*, which will be reported elsewhere, have shown that plasma derived from heated blood can produce a similar change in normal red cells.

Some of the patients studied showed partial thickness loss of an appreciable portion of the burn, notably Cases 6 and 13. A comparison of the curves obtained from Cases 6 and 7 demonstrates the results of this difference fairly well.

As was stated above, the involvement of the transfused cells seemed likely. This supposition was tested by Dr. Topley, who transfused M-negative blood to M-positive patients and separated the patient's cells from the transfused cells by a large-scale Ashby technique. Fig. 5 shows the results on Case 12, from which it is seen that the transfused cells are similarly affected.

A haemolysis technique was employed to obtain fractions enriched in older and younger cells. Using 0.4 g.% NaCl, approximately 90% of the cells remaining unlysed were 40 days old or younger (Marks *et al.*, 1958). The rates of alkali denaturation of these two fractions and those obtained by gravitational separation were tested. The results of both methods suggest that the younger age group of the cells may be preferentially affected.

Discussion

The temporary development of the abnormality indicates that the causative factor is most active during the first two days after injury. This is then followed by a gradual return to normal at a maximal rate of 5% (of the highest value) per day. As this exceeds the daily rate of red cell formation, it is probable that the affected cells are destroyed at least twice as rapidly as the normal cell population. The behaviour of Cases 6 and 7, which did not have a primary excision, but had very different areas of full-thickness skin loss, suggests that cells temporarily trapped in a partially damaged capillary bed, which would therefore suffer from transient anoxia, are more liable to be affected.

Fractionation experiments showed that the cells of low gravity and high saline resistance became more readily involved. It is well known that young cells are richer in enzymes than older cells, and, although Simon and Topper (1957) have shown that some young erythrocytes are also to be found among the most fragile cells, the method employed nevertheless yielded a fraction enriched in such cells. Levy, Walter, and Sass (1959) have shown that these young cells have the highest content of oxalacetic transaminase and lactic dehydrogenase. The conversion of glucose to lactate provides the major portion of the energy requirements of the cell. They are such that a high level of A.T.P. is required for the hexokinase reaction. Storage associated with oxygen lack causes a decrease in A.T.P. levels and hence a decrease in utilization of glucose. As the younger erythrocytes are metabolically the most active, at least part of the changes observed may be due to a premature ageing of some of the youngest erythrocytes.

Although the above hypothesis seems attractive, it must nevertheless not be overlooked that the method of fractionation would include cells which, although belonging to an older group, were rendered more saline resistant by exposure to unknown factors.

I should like to thank Dr. E. Topley for the M fractionation of patients' blood subsequent to transfusion and for much helpful and stimulating criticism.

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THE RELATIONSHIP OF SERUM GAMMA-GLOBULIN CONCENTRATION TO MALARIA AND SICKLING

BY

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The serum protein patterns of groups of Nigerians including 98 children partially protected by "daraprim" against malaria, and 113 unprotected children, have been studied.

The protected children have a significantly lower concentration of serum γ globulin than the unprotected children from the age of 12 months onwards.

In children, sicklers have a significantly higher serum γ globulin than non-sicklers.

It is suggested that sickling protects against malaria by enhancing the antibody response against the malaria parasite.

Surveys of serum protein patterns in different populations have shown that some communities have a higher mean concentration of serum γ globulin than others (van Oye and Charles, 1951; Holmes, Stanier, Semambo, and Jones, 1951; Arens and Brock, 1954; Vera and Roche, 1956; Edozien, 1957; Rawnsley, Yonan, and Reinhold, 1956; Nuñez Montiel, 1957; Bakker, Bliek, and Luyken, 1957; Curnow, 1957). Race, nutrition, and immune antibody response to certain endemic infections are some of the factors which are thought to be of significance in producing these differences. Of the endemic diseases malaria has been most frequently associated with high serum γ globulin. Holmes, Stanier, and Thompson (1955) in Uganda compared the protein patterns of three groups of Africans who are exposed to different rates of malaria transmission and suggested that a causal relationship may exist between malaria incidence and serum γ globulin level. McGregor, Gilles, Walters, Davies, and Pearson (1956) investigated a group of children, half of whom were protected for three years from malaria by weekly doses of chloroquine: they found significant differences in the γ globulin levels, the unprotected children having higher serum γ globulin than the protected children. In rats, Woodruff (1957) observed a significant increase in serum γ globulin six months after infection with *P. berghei*. In the present study the relationship between γ globulin and malaria prophylaxis is further examined in a group of Nigerian children after varying periods of protection.

Allison (1954) postulated that the sickle-cell trait afforded protection against subtertian malaria and that this advantage of heterozygote AS individuals over homozygous AA individuals was responsible for the maintenance of a high sickling rate in many populations in spite of the fact that homozygous SS individuals are continually being eliminated. There has been much controversy over the validity of Allison's conclusions and the mechanism by which this protection may be produced. A serious limitation in the investigation of this hypothesis is the difficulty of isolating a biological response of the body to the presence of the malaria parasite which can be measured and compared in sicklers and non-sicklers. Much of the work which has been done so far is centred around comparisons of infection rates, erythrocyte parasite densities, and infant mortality rates from malaria in sicklers and non-sicklers. Having established that one of the effects of malaria in a hyperendemic area is to produce a high mean serum γ globulin concentration, we consider that a study of the latter in sicklers and non-sicklers may throw further light on the relationship of sickling to malaria. We have therefore included in this study an examination of the relationship of sickling to serum γ globulin concentration.

Materials

The human subjects studied are (1) 350 Nigerians and 50 European adults at Ibadan (including 200 Nigerians and 40 Europeans previously reported (Edozien, 1958)); (2) 25 adults from Ilora, 35 miles

from Ibadan; (3) 40 patients with kwashiorkor aged 1½–2½ years from the villages near Ibadan; and (4) 211 children living at Imesi, a village 100 miles from Ibadan.

These 211 children are part of a group arising from 400 consecutive births in the village. The group has been studied from early in the mothers' pregnancies and it is hoped to follow the children through to their fifth birthday. One half of the mothers bearing these children were treated with pyrimethamine ("daraprim"), 50 mg. monthly, and the child when born has been given the same treatment, except that the children received 25 mg. a month. Daraprim tablets contain some lactose, and control tablets were made consisting entirely of lactose. The control tablets were used on the other half of the mothers and their offspring. The two tablets are indistinguishable in taste. If a mother or a child develops fever in either group they are given a single dose of chloroquine in the quantity advised by Covell, Coatney, Field, and Jaswant Singh (1955). The "daraprim" and control tablets were administered by the nursing sister who is resident in the village; the majority of the mothers have attended very well for their tablets and many of them have not missed one of their child's monthly tablets over two years.

Liver function tests on the lines previously described (Edozien, 1958) have been used to exclude liver disease in all the subjects included in this study.

Blood was collected by femoral vein puncture from the children and from an antecubital vein in adults. Part of each sample was placed in an oxalated bottle for the sickling test and paper electrophoresis of haemoglobin and the rest allowed to clot. The sera were separated on the day of collection, and the protein analyses on most of the specimens were performed on the same day. Sera which could not be handled on the day of collection were stored in the refrigerator at 4° C. and processed the following day.

Methods

Total Proteins.—The biuret method of Wolfson, Cohn, Calvary, and Ichiba (1948) was used.

Paper Electrophoresis of Proteins.—The method of Flynn and de Mayo (1951) was used. Serum, 6 µl., was applied to strips of Whatman No. 1 filter paper 2.5 cm. wide. Electrophoresis was for 18 hours at a constant current density of 0.5 ma. per strip and room temperature 23° C. ± 1° C. Barbitone buffer of *p*H 8.6 and ionic strength 0.083 was used. The papers were dried for 30 minutes at 110° C., then stained for 10 minutes in 0.75% azocarmine B (Gurr) in 50% methanol and 10% acetic acid and finally washed in successive changes of 10% acetic acid until a clear background was obtained. After drying at 110° C. for 15 minutes the papers were scanned in the "chromograph," an automatic reflectance scanner produced by Joyce, Loebl and Co., Newcastle, England. The protein fractions were computed as percentages of the total protein by dividing the area under each peak by the total area. By applying these percentages to the total serum protein the value of each fraction was obtained. No correction factors were applied: duplicate analyses show that values obtained in this way did not differ from values obtained by transmission densitometry with application of a globulin correction factor of 1.6 as previously reported (Edozien, 1958).

Sickling.—Slide sickling using 2% sodium metabisulphite was performed.

Paper Electrophoresis of Haemoglobin. — The samples were prepared for electrophoresis according to the method of Singer, Chernoff, and Singer (1951). Quantities, each of 25 µl., of an approximately 10% haemoglobin solution were applied from a micropipette to sheets of Whatman No. 3MM paper 20 × 36 cm., five samples and a control AS preparation being applied to one sheet. Barbitone buffer *p*H 8.6 and ionic strength 0.05 was used. Electrophoresis was for 10 to 12 hours at 230 volts and temperature 23° C. ±1° C. in a Shandon vertical tank. At the end of the run the sheets were dried in a hot air oven at 110° C. for 15 minutes.

Results

The Imesi subjects have been divided into two groups. Group A contains 98 children who received regular prophylactic doses of "daraprim" from birth. Group B is the control group, containing 113 children who did not get prophylactic antimalaria therapy although some of them were treated with chloroquine when they had fever. Within each group, the children have been further subdivided into three age groups: 4½–12 months, 12½–18 months, 18½–26 months. Table I shows the age distribution and haemoglobin genotype of the 211 children. In the analyses of the data, three children with γ globulin values of 2.55, 2.89, and 2.93 g./100 ml. have been excluded because these figures are well outside the range of all the other values and may be the result of unsuspected illness at the time the blood was collected.

TABLE I

HAEMOGLOBIN GENOTYPE OF THE 211 IMESI CHILDREN

	Age (Mth.)	AA	AS	SS	AC	CC	SC	Total
Protected	4½–12	35	9	1	0	0	0	45
	12½–18	16	5+1*	0	2	0	0	24
	18½–26	19	7	1	2	0	0	29
Unprotected	4½–12	32	3	2	2	0	0	39
	12½–18	30+1*	5	0	0	0	0	36
	18½–26	28+1*	8	0	1	0	0	38
	Total	161	39	4	7	0	0	211

* Excluded from analysis of data.

In Table II the serum proteins of protected AA and unprotected AA children are compared in order to show the effect of malaria prophylaxis on the serum proteins. It will be observed that there

are no significant differences in albumin, α_1 globulin, α_2 globulin, and β globulin concentrations between protected and unprotected children. Up to the age of 12 months no significant difference is found between the γ globulin concentrations of the two groups. At 12½–18 months and at 18½–26 months, however, there is a statistically significant difference ($0.02>P>0.01$ in either case) between the γ globulin concentrations of the two groups, the protected children having a significantly lower mean concentration of γ globulin.

Comparison of the serum proteins of unprotected AA and unprotected AS children and of protected AS and protected AA children are shown in Tables III and IV respectively. The mean γ globulin for the 13 unprotected AS children for the period 12–26 months of life is 1.79 ± 0.34 g./100 ml. as compared with 1.58 ± 0.32 g./100 ml. for the unprotected AA children of the same age. This difference is statistically significant ($0.05>P>0.02$). The difference between AA and AS individuals almost disappears if protected AS and protected AA children in the period 12–26 months are compared: the serum γ globulin for the protected AS children is 1.41 ± 0.28 g./100 ml. as compared with 1.35 ± 0.30 g./100 ml. for the AA children.

TABLE II
EFFECT OF MALARIA PROPHYLAXIS ON SERUM PROTEINS

	4½–12 Months (67)				12½–18 Months (46)				18½–26 Months (47)			
	Protected AA (35)		Unprotected AA (32)		Protected AA (16)		Unprotected AA (30)		Protected AA (19)		Unprotected AA (28)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Total proteins*	6·80	0·48	6·82	0·55	6·99	0·55	6·95	0·55	6·80	0·52	6·97	0·49
Albumin	3·25	0·40	3·18	0·35	3·29	0·38	3·14	0·38	3·23	0·34	3·25	0·37
α_1 globulin	0·30	0·15	0·32	0·13	0·29	0·13	0·35	0·15	0·29	0·11	0·29	0·12
α_2 ,,	1·07	0·28	1·02	0·21	1·10	0·26	0·87	0·25	0·99	0·19	0·92	0·18
β ,,	0·95	0·25	1·01	0·17	0·96	0·12	1·00	0·16	0·95	0·17	0·95	0·21
γ ,,	1·23	0·32	1·29	0·35	1·35	0·35	1·59	0·37	1·34	0·28	1·56	0·31

* All values expressed in g./100 ml.

TABLE III
SERUM PROTEINS OF UNPROTECTED AS AND AA INDIVIDUALS

	4½–12 Months				12½–18 Months				18½–26 Months			
	AS (3)†		AA (32)		AS (5)		AA (30)		AS (8)		AA (28)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Total proteins*	6·80	0·64	6·82	0·55	6·94	0·38	6·95	0·55	7·40	0·23	6·97	0·49
Albumin	3·39	0·38	3·17	0·35	3·19	0·40	3·14	0·38	3·23	0·18	3·25	0·37
α_1 globulin	0·24	0·11	0·32	0·13	0·26	0·12	0·35	0·15	0·42	0·09	0·29	0·12
α_2 ,,	0·88	0·14	1·02	0·21	0·87	0·18	0·87	0·25	0·97	0·12	0·92	0·18
β ,,	0·98	0·23	1·02	0·17	0·89	0·18	1·00	0·16	0·95	0·14	0·95	0·21
γ ,,	1·31	0·69	1·29	0·35	1·73	0·25	1·59	0·37	1·83	0·40	1·56	0·31

* All values expressed in g./100 ml. serum. † Figures in brackets represent the number of individuals investigated.

TABLE IV
SERUM PROTEINS OF PROTECTED AA AND AS CHILDREN

	4½–12 Months (44)				12½–18 Months (32)				18½–26 Months (26)			
	AA (35)		AS (9)		AA (16)		AS (5)		AA (19)		AS (7)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D
Total proteins*	6·80	0·48	6·70	0·39	6·99	0·55	6·96	0·39	6·80	0·52	6·90	0·31
Albumin	3·25	0·40	3·36	0·18	3·29	0·38	3·28	0·27	3·23	0·34	3·27	0·21
α_1 globulin	0·30	0·15	0·24	0·08	0·29	0·13	0·30	0·09	0·29	0·11	0·29	0·08
α_2 ,,	1·07	0·28	0·96	0·14	1·10	0·26	1·00	0·13	0·99	0·19	0·93	0·18
β ,,	0·95	0·25	0·95	0·18	0·96	0·12	0·98	0·15	0·95	0·17	0·95	0·16
γ ,,	1·23	0·32	1·19	0·39	1·35	0·35	1·40	0·39	1·34	0·28	1·42	0·23

* Values expressed in g./100 ml.

The analyses of the data from the Ibadan and Ilora subjects are presented in Table V and the serum protein changes in kwashiorkor are shown in Table VI. These results show that (1) the serum albumin of the group of Nigerian professional people is higher than that of Nigerians of lower economic status and approaches very close to the value in Europeans. (2) The mean serum gamma globulin of the professional group, although less than that of the non-professional group, is still significantly higher than the mean for Europeans. (3) The serum albumin of the village adults is only slightly lower than the level found in urban Nigerians who have the same or slightly better economic means, but the γ globulin level is very significantly higher. (4) Severe protein malnutrition (kwashiorkor) profoundly reduces the albumin level, but the changes in γ globulin are not significant. The conclusion drawn from these observations is that in health the serum albumin level is influenced by the nutritional status of the individual, and that the serum γ globulin, although subject to changes in socio-economic conditions, is independent of nutrition.

TABLE V

ANALYSIS OF DATA FOR IBADAN AND ILORA SUBJECTS

	Europeans in Ibadan (50)		Nigerian Professionals (50)		Non-professional Nigerians in Ibadan (300)		Ilora Adults (25)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Total proteins	6·92	0·53	7·27	0·47	6·87	0·40	8·24	0·60
Albumin	4·05	0·30	3·90	0·32	3·40	0·31	3·20	0·41
α_1 globulin	0·32	0·03	0·25	0·08	0·23	0·08	0·28	0·10
α_2 ,,	0·52	0·05	0·51	0·12	0·51	0·13	0·62	0·16
β ,,	0·83	0·12	0·80	0·18	0·68	0·18	0·97	0·19
γ ,,	1·20	0·21	1·81	0·31	2·05	0·35	3·17	0·45

TABLE VI

SERUM PROTEINS IN PATIENTS WITH KWASHIORKOR AND IN HEALTHY CHILDREN

	Healthy Unprotected Children Aged 18–26 Months (36)	Kwashiorkor Patients Aged 1½–2½ Years (40)	
	Mean (g./100 ml.)	Mean (g./100 ml.)	% of Normal Mean
Total proteins ..	7·06±0·50	4·13±0·64	58·5
Albumin	3·24±0·36	1·28±0·37	39·5
α_1 globulin ..	0·31±0·13	0·27±0·11	87·1
α_2 ,, ...	0·95±0·15	0·53±0·14	55·8
β ,, ...	0·95±0·18	0·41±0·19	43·2
γ ,, ...	1·62±0·38	1·64±0·51	101·2

Discussion

In discussing the results obtained in this survey we are fully conscious of the limitations imposed by our methods. The dosage scheme for "daraprim" was not intended to render the subjects completely parasite-free all the time. The village was organized by one of us (D.C.M.) for another clinical research project, and this dosage schedule was adopted because, while rendering the children free of parasites for most of the month, it still permitted a sufficient number of days of exposure to parasites to induce the development of natural immunity against malaria. Full details of infection rates and parasite densities in children protected according to this dosage schedule will be published elsewhere. This dosage scheme had the advantage that it cut down visits to the clinic to a minimum and this was responsible for keeping the number of defaulters to a negligible proportion. We believe that it is reasonable to presume that any differences found between the serum γ globulin levels of the two groups of children would have been greater had the children in group A been rendered free of parasites at all times.

Our data show that children who have received regular prophylactic doses of antimalarial drugs have lower mean γ globulin concentrations than children who have not been protected against malaria. The inference is that a high mean γ globulin concentration is caused, at least in part, by a response of the body to malaria parasites. We are, however, unable to indicate from our results the full extent to which malaria alone can account for the excess γ globulin in the serum of Nigerians, because our subjects were not completely parasite-free at all times. McGregor *et al.* (1956), who used weekly doses of chloroquine, found that the γ globulin values of protected Gambian children were still higher than in European children. Vera and Roche (1956), working in Caracas, Venezuela, where malaria infestation is at present a rarity, have observed an excess of γ globulin in the serum of negroes as compared with white subjects. Rawnsley *et al.* (1956), in a study of 45 Caucasoid and 45 North American negro blood donors of comparable social status in whom liver disease had been excluded, found a significantly higher concentration of serum γ globulin in the negro group. Schofield (1957), from a study of the serum protein pattern of West African students in London, reported that the γ globulin concentration did not fall to normal European values after several years of continuous residence in the United Kingdom. Bakker *et al.* (1957) reported high

γ globulin values in malaria-free natives of the Netherlands New Guinea. It is clear from these studies that there are other additional factors which contribute to the production of high γ globulin values in a community.

Our results support the view that nutrition is not an important factor in producing the hypergammaglobulinaemia in Africans (Holmes *et al.*, 1955; Rawnsley *et al.*, 1956; Comens, 1957). Curnow (1957) also found high γ globulin levels in Australian aborigines who are said to live on high-protein diets. Van Oye and Charles in 1956 re-examined the Africans of the Belgian Congo who were first investigated in 1951, and, despite improvements in nutrition standards which were represented in other blood values, no change was found in the serum globulin (van Oye and Charles, 1956).

Sickling may be a contributory factor since sicklers unprotected from malaria have a significantly higher concentration of γ globulin than unprotected non-sicklers. But, whereas hypergammaglobulinaemia appears to be universal in Africans, only 20–25% of adults in the Ibadan district sickle, hence sickling alone cannot explain all the facts.

Differences in the intensity of malaria transmission, together with the extent and efficiency of malaria control measures such as the use of mosquito nets and antimalarial drugs, may be partly responsible for differences in γ globulin level in groups of Nigerians. It must also be borne in mind that the standard of general sanitation and the quality of the water supply vary enormously, so that contact with other organisms such as water-borne pathogenic bacteria and parasites may be other contributory factors.

When all the evidence available at present is considered it points to the probability that the capacity to maintain a high serum γ globulin concentration in health is an inherited characteristic which has been selected for the protection it offers against malaria and possibly other endemic diseases. The actual levels found on examination depend on whether or not the individuals are in repeated contact with the organisms which cause the disease: thus Africans in hyperendemic malaria areas have higher values than Africans living in non-malarious areas, while the latter have higher values than Caucasoids living in comparable socio-economic circumstances.

Regarding the mechanism by which plasmodial growth can produce this effect, the suggestion has been made that the high γ globulin value is due to liver damage caused by malaria. A recent review of hepatic function tests in Nigerians (Edozien, 1958) has, however, failed to reveal any evidence of hepatic damage in symptom-free Nigerians even though they have high γ globulin values. The excess γ globulin was shown to have the flocculation properties of normal γ globulin, unlike the globulin in the serum in cases of hepatic disease, which behaves differently.

Holmes *et al.* (1955) have suggested a humoral mechanism. Our results are in agreement with this view. The response begins at about the age of 1 year. It is a reversible response, the γ globulin concentration decreasing when the stimulus is removed or diminished. However, until it becomes possible to measure quantitatively the amount of specific immune antibody in serum, we are unable to say categorically whether the excess γ globulin represents antibody or whether it is a by-product formed during the development of immunity to malaria.

The views currently expressed about the mechanism by which sickle haemoglobin protects against malaria relate principally to the existence of factors in the red blood corpuscles of carriers of the sickle cell trait which make it difficult for malaria parasites to establish themselves in sufficient numbers to cause serious illness (Allison, 1957; Raper, 1959). One hypothesis has been that sickle haemoglobin is unsuitable for the metabolism of the malaria parasite. Another theory is that changes occurring within the red blood corpuscles due to parasite metabolism cause the infected cells to sickle and are thereby removed from the circulation before the parasites can fully establish themselves (Miller, Neel, and Livingstone, 1956). In these, as in other hypotheses which have been put forward, the sickle haemoglobin, by preventing the establishment of malaria parasites in the red blood corpuscle, would be expected, like "daraprim," to be associated with a lower γ globulin concentration. Our results show that this is in fact not the case.

We therefore conclude that any protection which sickling may afford against malaria is not due to an inability of the malaria parasite to multiply and develop in the red blood corpuscle (a point confirmed by Raper's cultivation experiments). Our data, on the other hand, suggest that sickling may be associated with an altered or enhanced antibody response against the malaria parasite, since unprotected AS children have significantly higher mean γ globulin concentration than unprotected AA children of the same age, while the difference beween protected AS and protected AA children is not significant.

It would appear, therefore, that in the African the high serum γ globulin concentration to malaria and sickling are both directed to the same purpose, namely, protection against malaria and possibly other endemic diseases, but, while the γ globulin response has become universal, sickling has been limited because the homozygous SS state is lethal.

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THROMBOTIC THROMBOCYTOPENIC PURPURA

REPORT OF A CASE PRESENTING AS A CHRONIC NEUROLOGICAL DISORDER AND CHARACTERIZED BY UNUSUAL HISTOLOGICAL FINDINGS

BY

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A case of thrombotic thrombocytopenic purpura in a 50-year-old woman is described. Almost the whole course of the disease, lasting 18 months, was characterized by a bizarre neurological disorder, and the haematological manifestations first appeared at a late stage.

In many organs a vast number of arterioles and capillaries contained plugs of a fibrinoid material, and fibrinoid was subendothelially accumulated in a few of these vessels; but, in addition, medium-sized arteries of the myocardium were also obstructed by this same material. There were also verrucal endocardiosis of the mitral valve and slight thickening of the glomerular basement membranes. The striking diffusion of a pathological substance through damaged cerebral vessel walls into the nervous tissue seems to be a significant contribution to the understanding of the pathogenesis of the vascular pathology of thrombotic thrombocytopenic purpura.

Thrombotic thrombocytopenic purpura is still a rare disease (Frick and Hitzig, 1959), and the publication of another single case merits attention if new knowledge emerges. We report herewith a case distinguished by a chronic, remittent course lasting 18 months. The histological findings, though altogether typical, presented some peculiarities worthy of note.

The numerous synonyms given to this syndrome indicate some of the prevailing differences of opinion concerning the nature of the underlying pathological process, e.g., platelet thrombosis syndrome (Beigelman, 1951; Symmers and Barrowcliff, 1951), thrombotic microangiopathy (Symmers, 1956), thrombocytopenic purpura with vasothrombosis (Engel, Scheinker, and Humphrey, 1947), disseminated thrombocytic thrombosis (Wyatt and Lee, 1950), thrombotic acroangiothrombosis (Adams, Cammermeyer, and Fitzgerald, 1948). Craig and Gitlin (1957) speak of "hyaline thrombi" in thrombotic thrombocytopenic purpura, and Stuart and MacGregor-Robertson (1956) refer to it as a "hyperergic microangiopathy." The salient clinical and pathological features are amply recorded in the literature (Adams *et al.*, 1948; Gore, 1950; Hauser, Beyer, and Burger, 1951; Ladwig, 1953; Meacham, Orbison, Heinle, Steele, and Schaefer, 1951; O'Brien and Sibley, 1958). All the cases reported till 1959 had a fatal outcome. The early use of steroids and A.C.T.H. may be beneficial (Burke and Hartmann, 1959). The main clinical manifestations are fever, haemolytic anaemia, thrombocytopenia, purpura, and involvement of various organs with the relative exclusion of the lungs; the central nervous system and the kidneys are most often involved, but the myocardium (Frick and Hitzig, 1959), gastro-intestinal tract (Hellstrom, Nash, and Fisher, 1959), spleen (Gore, 1950), lymph nodes (Beigelman, 1951), and other organs are occasionally also clinically involved. The involvement of the central nervous system, which can appear at an early or late phase, is an almost constant feature. The complicated clinical picture is the sequel of widely disseminated arterioles and capillaries containing "thrombotic" lesions, which are associated with degenerative damage of the vessel walls and aneurysmal dilatation, particularly at the arteriolar-capillary junction (Orbison, 1952). The nature of the occlusive material is not yet established with certainty.

Case Report

H. H., a 50-year-old housewife, mother of a healthy girl of 14, suffered since her youth from attacks of headache accompanied by nausea and vomiting, occurring several times a year. Her present illness

began suddenly; while at work, she experienced a sudden, severe headache, vomited, and felt weakness of the left upper limb. For about 10 minutes her speech was unintelligible. A few hours later she noticed weakness of the left lower limb too. The patient was admitted to another hospital, where left hemiplegia was diagnosed. Her condition improved rapidly, but movements of the left hand and fingers remained somewhat impaired. Her speech was unaffected. The blood pressure was 140/95 mm. Hg. The blood picture and urine were normal.

Four months later, a sudden speech disturbance occurred, which, according to the description of the patient's relatives, was interpreted as a motor-sensory aphasia. After a few days only slight verbal aphasia remained, but the patient's mental demeanour changed; she became irritable, weepy, and forgetful. Shortly thereafter she once more experienced an attack of headache accompanied by vomiting and speech disturbance. The patient was referred to the neurological department of the Beilinson Hospital.

Examination revealed a pale undernourished woman not in acute distress. She spoke remarkably slowly and had great difficulty in finding the right words, but she was well orientated and gave a clear anamnesis. Her body temperature was 100° F., blood pressure 140/90 mm. Hg, pulse rate 96 per minute. No abnormalities of the thoracic and abdominal organs were found. A left hemiparesis, more pronounced in the upper limb and especially involving the distal parts, was noted. The muscles were hypertonic and the reflexes were hyperactive bilaterally, but more so on the left side. The left abdominal reflexes were absent. Babinski's, Oppenheim's and Chaddeck's signs were present on the left side. The right plantar reflex was not elicitable and Babinski's sign was found at times. Sensation was intact. The pupils and eye grounds were normal. *X*-ray examination of the skull showed no abnormality. Laboratory findings were as follows: sedimentation rate 18 mm. per hour (Westergren), haemoglobin 8.6 g. per 100 ml., red blood cells 2,900,000, white blood cells 6,200; the platelets were not counted, but mentioned as being normal; blood sugar was 96 mg. per 100 ml., urea 30 mg. per 100 ml. Analysis of urine was negative. The cerebrospinal fluid was clear, its pressure normal, the Pandy test negative, protein 36 mg. per 100 ml., sugar 88 mg. per 100 ml. On the electroencephalographic tracings slow waves (3–6 per minute) of low voltage were recorded in the anterior and middle regions of the left temporal lobe. The family declined ventriculography. During the following days the patient's condition improved considerably. Because of the anaemia iron therapy was instituted. The patient was discharged in a satisfactory state; the haemoglobin was 11.3 g. per 100 ml., red blood cells 3,450,000 and platelets 180,000.

At home the patient's condition deteriorated gradually and necessitated admission to hospital for the third time, 10 months after her last discharge. According to the history given by the family, she went through repeated episodes characterized by headache and vomiting, disturbances of speech and memory. Several times convulsive movements of the left body side, and later also of the right side, were observed. From time to time the patient was febrile. On the day before admission, a sudden right paralysis and complete loss of speech occurred.

Examination disclosed a right hemiplegia, hypertonic muscles, and pathological reflexes. A complete motor-sensory aphasia was noted. Testing of sensation was uncertain. The Rumpel-Leede phenomenon was positive. The blood pressure was 140/90 mm. Hg. The eye grounds were normal. Lumbar puncture revealed clear fluid under raised pressure and containing 6 cells per c.mm., the Pandy test was negative, protein 49 mg. per 100 ml. Laboratory findings were as follows: sedimentation rate 19 mm. per hour (Westergren), Wassermann reaction negative, urea 48 mg. per 100 ml., haemoglobin 9.7 g. per 100 ml., red blood cells 3,800,000, white blood cells 11,650 with a normal differential, platelets 37,000. The bleeding time was three and a half minutes and coagulation time seven minutes. A red blood cell fragility test gave the following results: haemolysis began at a concentration of 0.48% NaCl and the process was complete at a concentration of 0.25% NaCl. Urine analysis disclosed up to 30 fresh red blood cells per high-power field and many granulated casts in the deposit. In the days following, the patient's course went rapidly downhill, many cutaneous petechial and ecchymatous haemorrhages appeared all over the body, and blood examination revealed progressive anaemia (haemoglobin 7.6 g. per 100 ml., red blood cells 2,700,000, haematocrit 23% (reticulocytes 16‰ per c.mm., platelets 30,000). The patient lapsed into coma and expired 48 hours later.

Necropsy Findings

The body was that of a thin, middle-aged-looking woman. There was no jaundice. Many cutaneous and deep subcutaneous petechial and ecchymatous haemorrhages were scattered all over the body.

The left pleural cavity contained a serous effusion of 200 ml. The lungs were emphysematous, congested, and oedematous. There were moderate tracheobronchitis and bronchopneumonic foci in both lower lobes. The heart was of normal size (340 g.) and configuration. Subendocardial petechial haemorrhages were found in the left ventricle. The myocardium of both ventricles was marked by numerous tiny haemorrhages. The anterior wall of the left ventricle contained numerous scars, 1 to 2 mm. in diameter. On the dorsal cusp of the otherwise normal-appearing mitral valve a small group of tiny, soft, and reddish vegetations was situated. The coronary arteries were within normal limits, as were all the great body arteries. The alimentary

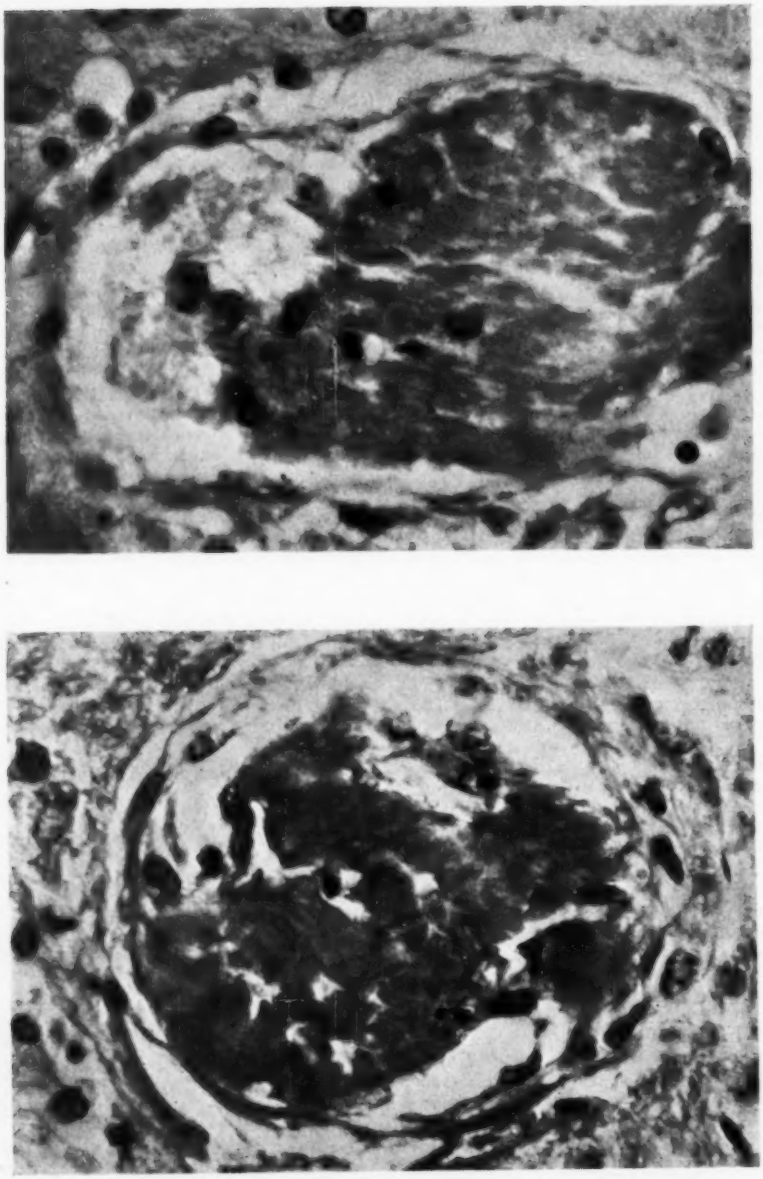

FIG. 1.—The typical lesion of thrombotic thrombocytopenic purpura. Dilated cerebral arterioles containing plugs of a smudgy and granular, eosinophilic material, within which a few cells are enmeshed. Haematoxylin and eosin. × 360.

tract was intact. The liver weighed 1,530 g., and its lobular pattern was accentuated due to a moderate degree of centrilobular congestion. The spleen was small (80 g.), its parenchyma normal. The pancreas contained small haemorrhages, which were scattered here and there in groups. The kidneys were rather small (combined weight 230 g.), the capsules stripped with ease, the surfaces were irregular, the cortices were pale, the cortico-medullary junction was distinct. Many petechial haemorrhages were scattered throughout the cortices. The pelvices, ureters and urinary bladder were normal. The adrenal glands showed nothing of note. The internal genital organs were atrophic.

The brain was anaemic and oedematous. The walls of the larger arteries were delicate and the lumina were patent. Coronal sections disclosed numerous old and recent, variously sized and shaped encephalomalacic foci in both cerebral hemispheres. The majority were located in the cortical and subcortical areas, but some were also found elsewhere, namely, in the white matter of the left frontal, right temporal and left occipital lobes, and on the right side in the internal capsule, putamen, caudate nucleus, corona radiata, and surrounding the posterior cornu of the lateral ventricle. No lesions were present in the cerebellum, pons, and medulla oblongata.

Microscopic Examination

The outstanding histological finding was partial or complete occlusion of an excessive number of arterioles and capillaries by plugs consisting of a smudgy, granular, eosinophilic, periodic-acid-Schiff-positive and argyrophilic (Gomori's method) material, in which no fibrin could be demonstrated (henceforth to be referred to as "thrombotic" material). There was no inflammatory infiltrate associated with these lesions. Fig. 1 illustrates, for example, a typical lesion in the brain. The material either lay in the lumen unattached to the vessel wall in the plane of section or it was attached to the wall; sometimes it was covered by a layer of endothelial cells and/or contained endothelial cells or fibroblasts within it, and rarely it was replaced by a loose connective tissue. Some of the vessels were markedly dilated. In relatively few vessels there was a subendothelial accumulation of hyaline, eosinophilic, periodic-acid-Schiff-positive material, at times blurring the structure of the wall. This was either localized to a part of the circumference or encircled it completely. The subendothelial deposit was either in continuity with an intraluminal plug or it occurred independently.

Kidneys.—Many glomeruli were intact, but others showed a slight thickening of the basement membranes of the capillary walls and of the basement membranes of the Bowman's capsules. Many of the vasa afferentia, but only relatively few glomerular capillaries, vasa efferentia and arterioles and capillaries of the interstitial tissue contained "thrombotic" material. Fig. 2 demonstrates such a finding, involving the afferent and efferent arterioles as well as some capillaries of one glomerulus. Many proximal tubules were dilated and their epithelial cells contained accumulations of hyaline droplets. Hyaline casts, which gave a positive Prussian blue reaction, were present in the tubules.

FIG. 2.—Plugging of the afferent and efferent arterioles in the kidney as well as some glomerular capillaries. Dilated proximal convoluted tubules. Haematoxylin and eosin. × 190.

FIG. 3.—Patchy fibrosis of the myocardium. A medium-sized artery and some arterioles contain "thrombotic" lesions. Haematoxylin and eosin. × 40

Heart.—Fig. 3 shows a representative section of the ventricular myocardium. The continuity of the muscle fibres was disrupted by areas of scar and granulation tissue of various sizes and shapes. Many muscle fibres were hypertrophic. In the septal myocardium foci of recent infarction were seen. Numerous arterioles and capillaries contained "thrombotic" material and a few were occluded by fibroblastic proliferation. In contradistinction to the other organs, also medium-sized arteries were involved in the process (Figs. 3 and 4); their lumina were partially or completely obstructed by connective tissue and/or the "thrombotic" mass. Fig. 4 illustrates a medium-sized artery, the lumen of which was narrowed by a signet-ring-shaped fibrous thickening of the intima, endothelialized except on its summit, to which was adherent a polypous structure composed of a smudgy, eosinophilic, argyrophilic and periodic-acid-Schiff-positive material devoid of fibrin (as demonstrated by the Weigert's and phosphotungstic-acid-haematoxylin stains); it contained a few fibroblasts and was partly endothelialized.

The vegetations on the dorsal cusp of the mitral valve, which was focally hyalinized, consisted of material morphologically and tinctorially indistinguishable from that contained in the blood vessels.

Lungs.—There were intra-alveolar haemorrhages and large accumulations of alveolar phagocytes containing haemosiderin. "Megakaryocyte embolization," as demonstrated in the capillaries of the interalveolar septa by the P.A.S. stain (Sharnoff and Kim, 1958), was extensive. No vessels containing "thrombotic" material were found.

Pancreas.—Many small foci of acute pancreatic necrosis, associated with arterioles and capillaries containing "thrombotic" lesions, and recent interstitial haemorrhages were scattered throughout the organ. The capillaries of the Langerhans' islets were heavily involved and here and there an intrainsular haemorrhage was noted.

Liver.—Acute passive congestion, a few "thrombotic" lesions, and haemosiderosis were observed.

Spleen.—There were acute passive congestion, swelling of the littoral cells, a few "thrombotic" lesions, periarteriolar fibrosis and haemosiderosis.

Lymph Nodes.—The lymphatic component was decreased in amount and there were no germinal centres; a few "thrombotic" lesions and a remarkable degree of haemosiderosis existed.

Adrenal Glands.—In the zona glomerulosa there were numerous "thrombotic" lesions; the zona fasciculata, on the other hand, was the site of small interstitial haemorrhages.

Oesophagus, Thyroid Gland, and Skeletal Muscle.—Only very few blood vessels contained "thrombotic" lesions.

Skin.—No "thrombotic" lesions could be demonstrated, though the cutaneous and subcutaneous tissues were sprinkled by extravasated blood cells.

Bone Marrow.—The erythropoiesis was active; the megakaryocytes were increased in number. A few macrophages contained haemosiderin. There were no vessels containing "thrombotic" material.

Brain.—The brain was markedly oedematous; in different areas there were lesions ranging from most recent ischaemic infarction and diffuse

or perivascular haemorrhages to older foci of malacia characterized by the appearance of scavenger cells and reactive gliosis. In the oedematous regions and in the meninges there were only a few blood vessels containing "thrombotic" material. On the other hand, in the severely damaged tissue, as well as in the choroid plexus, the "thrombotic" lesions were numerous. It should be emphasized that in the older foci of malacia only few vessels were obstructed by fibroblastic proliferation, whereas most of the involved arterioles and capillaries contained unaltered "thrombotic" material. In multiple sections from oedematous regions, diffusion of a periodic-acid-Schiff-positive material through the vessel walls was found (Fig. 5). Small blood vessels, the walls of which were slightly thickened due to inhibition by periodic-acid-Schiff-positive material, were surrounded by confluent, roundish to polygonal masses of a periodic-acid-Schiff-positive substance infiltrating deep into the adjacent nervous tissue. This unusual picture reminded one to a certain extent of the so-called "grape-like bodies" of Buscaino (Jakob, 1927). Corresponding sections, stained with phosphotungstic-acid-haematoxylin, revealed an almost complete absence of the neuroglia in those places where the periodic-acid-Schiff-positive material was deposited (Fig. 6).

In the hypophysis, the blood vessels of the anterior lobe appeared normal, whereas many arterioles and capillaries of the posterior lobe contained "thrombotic" lesions.

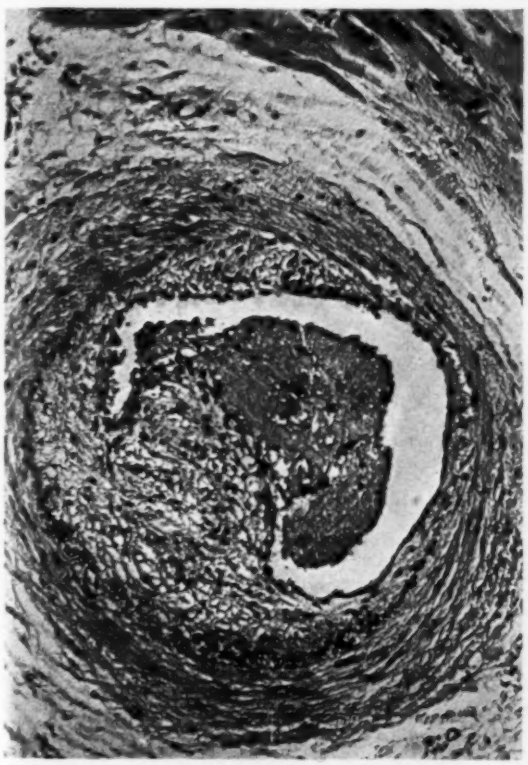

FIG. 4.—Narrowing of the lumen of a medium-sized artery in the heart by a signet-ring-shaped fibrous intimal thickening, to which is attached a "thrombotic" polypous structure. Haematoxylin and eosin. × 100.

Comment and Further Investigation

Several features of the presented case are apparently atypical and unusual. The patient's illness, masquerading as a neurological disorder, lasted 18 months and the haematological manifestations appeared only terminally. Histological evidence of involvement of medium-sized arteries in the heart and verrucal endocardiosis (Allen and Sirota, 1944) were found. Middle-sized arteries containing "thrombotic" lesions were also observed by Cooper, Stickney, Pease, and Bennett (1952), and vegetations on the cusps of the aortic valve were present in one of Beigelman's (1951) cases and on the cusps of the mitral valve in the case reported by Engel *et al.* (1947). In the brain there was diffusion of a periodic-acid-Schiff-positive material through damaged vessel walls into the nervous tissue. The diagnosis of thrombotic thrombocytopenic purpura in this case is based on the clinical picture of an intermittent bizarre neurological disorder associated with fever, anaemia, thrombocytopenia, and purpura; the microscopic picture was characterized by the presence of widely disseminated arterioles and capillaries containing a smudgy, granular, eosinophilic material and a degenerative alteration of the vessel walls, i.e., subendothelial accumulation of hyaline eosinophilic material.

Accepting the case as an authentic example of thrombotic thrombocytopenic purpura, it seems to us that verrucal endocardiosis and involvement of medium-sized arteries can be part of the syndrome. The question arises whether the material found in these arteries is identical to or different from that present in the arterioles and capillaries. Because the exact composition of the "thrombotic"

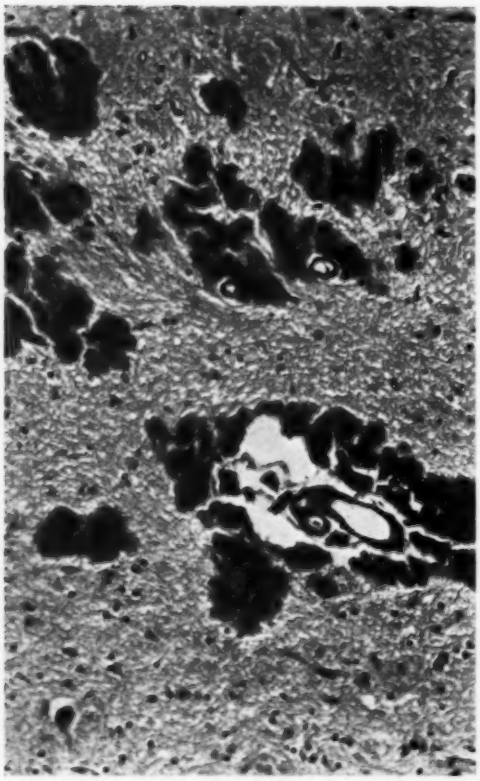

FIG. 5.—In the brain, surrounding slightly thickened arterioles and capillaries, there are confluent, roundish to polyhedral, periodic-acid-Schiff-positive masses. Periodic-acid-Schiff. × 80.

material can be defined neither morphologically nor on staining, we subjected the lesion of medium-sized arteries to digestion with trypsin.

Serial sections of the area illustrated in Fig. 4 were cut and alternating sections were submitted to tryptic digestion. The other sections were used as controls. After deparaffination and hydration, the slides were immersed in a 0.1% solution of trypsin in 0.01 M phosphate buffer of *p*H 7.6 respectively in the buffer solvent. The slides were removed at various intervals up to a total of 19 hours, i.e., after one, two, three, four, six, 10, 14, and 19 hours, and examined immediately after staining by the periodic-acid-Schiff technique. As was previously described by Fisher and Creed (1955), the "thrombotic" lesions in the arterioles and capillaries retained their positivity to the periodic-acid-Schiff reaction. It was our aim to evaluate the lesions in the medium-sized arteries; the eosinophilic material attached to the fibrous intimal cushion of the artery illustrated in Fig. 4 was completely stained by the periodic-acid-Schiff reaction before beginning the experiment as well as in all control sections. No changes were observed in the first three sections removed from the trypsin solution. In the section removed after the fourth hour of digestion some material was dissolved out of the "thrombotic" mass. Fig. 7 illustrates within the lesion a small, roundish, sharply demarcated area, which became negative to the periodic-acid-Schiff reaction. All the succeeding sections, i.e., up to the nineteenth hour of digestion, disclosed exactly the same picture; only this distinct area lost its stain uptake by the periodic-acid-Schiff reaction.

It is well known that agglutinated platelets, fibrin, and fibrinoid cannot be differentiated conclusively by their tinctorial behaviour. In our case, and in that reported by Meacham *et al.* (1951), the lesions under discussion contained no

FIG. 6.—Absence of the neuroglia in the vicinity of the blood vessels. Phosphotungstic acid haematoxylin. × 80.

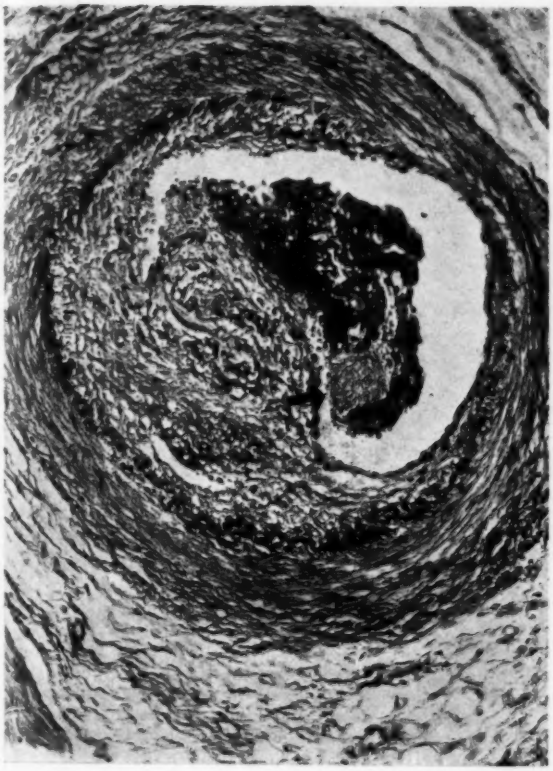

FIG. 7.—Same artery as illustrated in Fig. 4. Periodic-acid-Schiff stain after tryptic digestion. The arrow points out a small area, which became negative after the fourth hour of digestion. × 100.

fibrin demonstrable with the Weigert's and phosphotungstic-acid-haematoxylin stains, but other authors found small amounts of fibrin within the "thrombotic" masses (Symmers and Barrowcliff, 1951; Cooper *et al.*, 1952). According to Fisher and Creed (1955), fibrinoid material, contrary to platelets and fibrin, is not digestible by trypsin. It is our opinion that the available evidence allows the conclusion that the "thrombotic" lesions in the small as well as in the medium-sized vessels consist chiefly of a material identical with, or at least closely related to, fibrinoid, but, on the other hand, they contain a small amount of agglutinated platelets and/or fibrin. The failure to demonstrate this relationship in the arterioles and capillaries containing "thrombotic" lesions may be due to a more thorough combination of fibrinoid and platelets and/or fibrin in the small vessels, thus making the recognition of minimal digestible amounts impossible with the magnifications obtainable with the light microscope.

Discussion

In the brain and heart recent ischaemic infarctions, reparative processes, and scarring were found. It is, therefore, significant to call attention to the fact that only few arterioles and capillaries were occluded by fibroblastic proliferation, whereas the majority of the vessels contained unaltered "thrombotic" lesions. Hence it follows that the vascular lesions of thrombotic thrombocytopenic purpura, in spite of long duration, which was at least 18 months in our case, are distinguished by a limited tendency to organization. It may be assumed that the pathological changes described in the cerebral vessels preceded the clinical manifestations by a certain period of time, insofar as in relatively large brain areas only the successive alteration of a vast number of arterioles and capillaries could cause the foci of malacia. It was Gore (1950) who established the fact that the pathological process is "episodic and occurs in crops." With the progression of the cerebral lesions, signs of participation of both hemispheres became evident. It is noteworthy that, in spite of the involvement of many organs, the illness of our patient was characterized, except terminally, only by a neurological disorder.

Our observations are in accord with the opinion of other authors (Fisher and Creed, 1955; March, 1954) that the "thrombotic" lesions conform to the reactions of fibrinoid, but, in consistency with our investigations, we believe that fibrin and/or platelets take part in their formation. It was recently proved that fibrinoid is composed principally of fibrin (Gitlin, Craig, and Janeway, 1957), and Craig and Gitlin (1957), using fluorescein-labelled antisera, showed that the "thrombotic" material in thrombotic thrombocytopenic purpura is composed of fibrin or an insoluble derivative of fibrinogen.

In the literature available to us we did not encounter the description of diffusion of periodic-acid-Schiff-positive material into the nervous tissue. With regard to the divergence of views in the literature (Jakob, 1927) concerning structures of such a kind, it is our opinion that the observation does not reflect an artifact, but rather that the same material, which was present as plugs in the lumina of the blood vessels and as a

subendothelial accumulation, penetrated into the nervous tissue through the damaged vessel walls and diffused into the surrounding tissue.

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STRESS ZONES IN FOETAL ARTERIES

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In a study of the peripheral arteries of foetuses it was found that focal areas of intimal thickening and of splitting of the internal elastica had developed, the thickening being similar to that found in the coronary arteries. Characteristically the thickenings were found in the mouths of branches and in those vessels subject to pulsation. It is believed that they are physiological in nature and a response of the vascular wall to the stresses produced by focal areas of impaired pulsation and not degenerative as formerly thought.

The evolution of these arterial cushions is traced from early foetal life; their most important constituent is bundles of medial longitudinal muscle. Both thickening and splitting of the internal elastica are brought about by a radial reorientation and migration of the muscle cells of the media.

Material

The vessels were obtained from 25 foetuses, of which 14 were male and 11 female. Their maturity ranged from 30 weeks' gestation to full term. All of them died within 24 hours of birth except one, a premature infant of 35 weeks' gestation who lived for four days after birth. In addition, the vessels of four early foetuses of from 14 to 20 weeks' maturity were also examined.

A small segment of each artery studied was taken from the same site in each case. In the upper limb these comprised the brachial artery, both at the level of the insertion of the deltoid muscle and in the antecubital fossa, as well as the radial artery at the wrist. In the leg, the vessels examined were the femoral artery, in the upper part of the subsartorial canal, the popliteal, in the popliteal fossa, and the posterior tibial artery from just above the medial malleolus.

From each of these short segments three blocks were taken more or less at random, although, if a branch were present, then its mouth was included in one of the blocks. Several sections were also taken from the abdominal aorta in each case to show the mouths of the lumbar vessels. In eight cases random blocks were taken from the coronary arteries for comparative purposes. All the sections were stained with haematoxylin and eosin and with Weigert's elastic tissue stain and van Gieson. Some were also stained with Sudan IV and haematoxylin for lipoid.

Findings

Peripheral Arteries.—In most of the peripheral vessels the intima consisted only of a layer of endothelium lying directly on the internal elastic lamina. In some, however, there were focal areas of thickening which varied from a simple splitting of the internal elastic lamina to the formation of large cushion-like structures which projected into the lumen.

These arterial cushions had a characteristic distribution in the peripheral vessels as well as a distinctive location in their walls. They were found only in the popliteal and lower end of the brachial artery. Most often they occurred at the margins of branches and in the wall of the parent vessel opposite the mouth of the branch (Fig. 1). However, they were not restricted to these sites and, especially in the popliteal artery, they were also found in places where there was no evidence of branching. Nevertheless, there tended to be a pattern in their distribution in that they often occurred in groups, several being present in one segment of the vessel. There was a marked difference both in the size and frequency of cushions in the popliteal and brachial arteries. They were found in the popliteal vessel in 76% of the cases and here attained a much larger size than they did in the lower end of the brachial artery where they were also less frequent.

The structure of the cushions was variable, but they were all musculo-elastic in nature, were generally symmetrical in shape and formed a definite thickening of the vessel wall. The great majority of them had both an intimal and a medial component. In the media there occurred

FIG. 1.—Popliteal artery in a female at full term, showing the characteristic location of arterial cushions. Weigert, × 70.

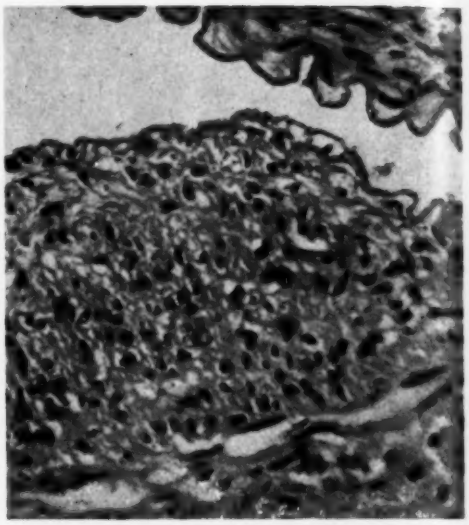

FIG. 2.—Popliteal artery in a female of 30 weeks' gestation. A cushion showing the longitudinal direction of its muscle. Haematoxylin and eosin, × 350.

FIG. 3.—Popliteal artery in a male, full term. The internal elastica can be identified. Below it the elastic network of a medial longitudinal muscle band is prominent. In the intima longitudinal muscle lies in an elastic mesh derived from the elastica. Weigert, × 250.

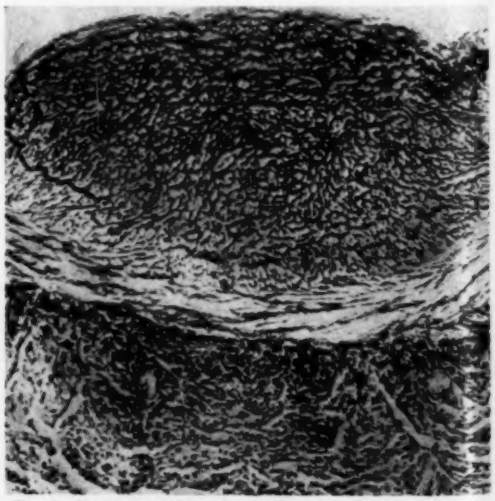

FIG. 4.—Popliteal artery in a female, full term. The internal elastica has become greatly interrupted and lies beneath a mound of elastic fibrils and longitudinal muscle. A thin rim of longitudinal medial fibres can be seen. Weigert, × 175.

bundles of longitudinally running muscle fibres which lay in a feltwork of fine elastic filaments so intimate that almost every muscle fibre was invested by it (Figs. 2 and 3). The intimal portion was formed by a splitting of the internal elastic lamina, sometimes in a fibrillary fashion and sometimes into several coarse lamellae between which longitudinal muscle was also present (Fig. 3). In occasional cushions the intimal element was predominant so that remnants of the internal elastica could be clearly seen beneath a large mound of longitudinal muscle and elastic tissue, while in the media only a thin rim of longitudinal fibres could be distinguished (Fig. 4). More often, however, instances were found in which a medial component alone was present. This took the form of a short band of longitudinal muscle which produced no thickening of the wall and lay immediately below an internal elastic lamina which was intact or showed only the earliest stages of splitting (Fig. 5). Transitions between these forms and the more complex cushions frequently occurred.

Although, as described, these cushions were mainly composed of longitudinally running muscle some radially directed fibres were also observed. These occurred especially on the deep surface of a cushion and in places where longitudinal muscle bundles in the media appeared to be forming and extending laterally in the vessel wall. Here the circular fibres of the media could be seen to sweep round in a radial fashion and to merge with the longitudinal muscle. Quite often also, radial fibres were seen which appeared to be squeezing themselves through small gaps in the internal elastic lamina.

It is emphasized that the cushions described above were essentially musculo-elastic structures and that the vast majority of them showed either no evidence of a fibrous tissue component or contained but a few strands of it running alongside the elastic fibrils of the thickening. Only occasional cushions were quite definitely fibrous. In these the elastic tissue tended to be more finely divided and the fibrous tissue occurred in the superficial parts of the thickening amongst the mesh of fine elastic filaments.

Deposits of lipoid were not a characteristic feature of these arterial cushions. In seven of the cases serial frozen sections of the popliteal artery were stained with Sudan IV and haematoxylin for lipoid. In this way, three or four different cushions from each case were studied. In only one, a premature infant of 35 weeks' gestation who survived for four days after birth, was any lipoid detected. In this case every cushion was involved, and the lipoid occurred as a fine, diffuse deposit in the superficial parts of the thickenings. In the sections stained with haematoxylin and eosin the appearance of only three cushions suggested that they might contain lipoid. In only one of them was there any fibrous tissue, the other two being purely musculo-elastic in structure.

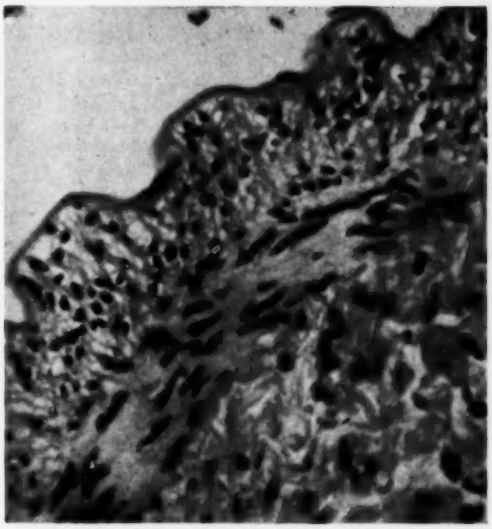

FIG. 5.—A band of longitudinal muscle lies below the internal elastica which shows no evidence of reduplication. Haematoxylin and eosin, × 400.

Aorta.—In contrast to the peripheral vessels the aorta showed little evidence of the formation of muscular thickenings at the margins of its branches. In the section stained with Weigert's elastic stain there was no evidence of complex elastic tissue changes similar to those which threw the cushions of the peripheral arteries so readily into relief. A close examination of the sections stained with haematoxylin and eosin, however, did show that there was a definite tendency for the muscle cells in the aortic wall to be longitudinally arranged at the margins of branches. In comparison with the changes in the peripheral arteries those in the aorta were quite unspectacular and were present at the mouths of only a proportion of the branches.

Arteries of the Early Foetus.—In an attempt to trace the earliest beginnings of arterial cushions, the peripheral vessels of four early foetuses of 14, 16, 18, and 20 weeks' gestation

were examined. The 16-week foetus was the earliest one in which there was a suggestion of the formation of muscular cushions. They occurred as slight thickenings of the wall of the popliteal artery which increased in both size and number during the eighteenth and twentieth weeks. Their structure was at first simple and consisted solely of a small node of longitudinally orientated muscle with no evidence of reduplication of the overlying internal elastic lamina. It was not until the twentieth week that this lamina split. At this time too, the longitudinal muscle of the thickening began to develop its intrinsic elastic net (Fig. 6).

Coronary Arteries. — In view of previous reports on the finding of intimal cushions in the coronary arteries of the newborn, it was thought necessary to compare the thickenings already described with those occurring in these vessels. Accordingly, the coronary arteries were examined in eight cases. These comprised six male and two female foetuses, all of which were stillborn save one infant who survived for four days after birth. Random sections were taken from both the anterior descending and the circumflex branch of the left coronary artery.

FIG. 6.—Popliteal artery in a 20-week foetus. Bundles of longitudinal muscle are present at the margins of a branch. The internal elastica shows early reduplication. The gap in the lamina over the left cushion is occupied by radial smooth muscle cells. Weigert, × 125.

In four of the infants, the two female and two of the male, no coronary cushions were found. In the other four structural changes were present which were similar to those found in the peripheral arteries. They differed from those in the popliteal artery only in that they were generally smaller and more widely scattered in the coronary vessels. The only coronary cushion in which lipoid was found came from the heart of the premature infant of 35 weeks' gestation who lived for four days after birth. There was no evidence in the van Gieson stain of a connective tissue component in this cushion. It is interesting to note that it was this same infant which was the only one to show the presence of a diffuse infiltration of lipoid in the frozen sections of the popliteal cushions.

Discussion

In 1946, Dock reported the finding of intimal cushions in the coronary arteries of the newborn, but did not go into any histological detail. He found that the thickenings were more marked in male than female infants, and considered that their presence was perhaps related to the coronary vessels having to adapt themselves to a changing length during cardiac systole. Hence, the males, with their slow pulses, high stroke volume, and greater changes in length of the vessels, were more severely affected. Dock believed that thus was established the predilection of atherosclerosis for the coronary vessels and its greater incidence in males than females.

The presence of these cushions in the coronary arteries of infants was later confirmed by Fangman and Hellwig (1947), Lack (1947, cited by Anderson, 1953), Minkowski (1947), Levene (1956), and Moon (1957). However, these later authors differed from Dock in the interpretation of their significance. Thus, Fangman and Hellwig believed that they represented the earliest stages of arteriosclerosis, that a deposition of lipoid was the primary event in their formation and that this then resulted in a degeneration of elastic tissue. Both Levene and Moon also believed these coronary cushions to be pathological. The former held that they represented sites of underlying medial weakness and the latter that they were the early phases of the arteriosclerotic process. Minkowski, on the other hand, was more cautious. While recognizing that the thickenings showed a strong resemblance to early

adult arteriosclerosis, he considered that their real significance had yet to be clarified. Lack is stated by Anderson to have found that the thickenings were confined to points of branching tapering off above and below these levels, but no opinion is given as to their nature.

The findings of the present study do not support the view that these cushions are either primarily intimal in origin or degenerative in nature. Thus, as has been described, from their inception in early foetal life and throughout their development, the most important constituent of these thickenings is a bundle of longitudinal medial muscle. Indeed, when first formed, they consist solely of longitudinal muscle. It is only during their later development that there is a progressive splitting of the overlying internal elastica and the acquirement, by a proportion, of connective tissue and occasional foam cells. The very appearance of the cushions is opposed to the theory that they are degenerative in nature. There is a symmetry and even a suggestion of architectural design in the interweaving of their smooth muscle and elastic tissue which suggest purpose and function.

Previous workers have stressed the similarity in the distribution of the cushions in the coronary arteries to that of atherosclerosis and have put this forward as further evidence of their close relationship to this disease. However, when their presence also in the popliteal and antecubital fossae and their peculiar tendency to localize in certain situations in these vessels is considered, then a different explanation of their significance becomes probable.

In attempts to explain the tendency of arteriosclerosis to localize at branchings, it has long been suggested that pulsation gives rise to important haemodynamic stresses in these regions (Duguid, 1926; Moschcowitz, 1942). Duguid especially has emphasized the systolic elongation in vessels during pulsation, and believed that in regions of branching the branch acted as a "drag," tending to fix the parent vessel, resulting in a damaging strain to its wall. This tendency to elongate as well as dilate during systole is often forgotten, but that it is an important effect of pulsation is evident on consideration of the fibrosed arteriosclerotic peripheral vessels of the elderly which are not only markedly dilated but are also tortuous.

If a branch does impair the systolic elongation of the parent vessel, perhaps by the tone and elasticity of its muscular coat and by the interlacing of its adventitial coat at the point of junction, then at each pulse beat there would be a tugging at the margins of the branch which would be longitudinal in direction. In the peripheral arteries, where the muscular coat is composed of circular muscle fibres, such a longitudinal stress cannot be effectively countered and would eventually be likely to lead to a disruption of the wall at this point. It is suggested, therefore, that the cushions of foetal arteries are but the physiological reaction of the vessel to such a longitudinal stress and that by the tonus and elasticity of their muscle they prevent tearing and damage to the arterial wall. The muscular cushions found in some cases opposite the mouths of branches as well as at their margins are probably due to similar causes. Here, it would seem, the interweaving of the muscular and adventitial coats of the parent vessel and branch is such that it leads to fixation of the whole segment of the parent artery and not of just the region around the orifice of the branch. In the same way, the presence of any local factor such as the proximity of an artery to bone, its crossing by a fascial band or tendon, or the presence of a neighbouring actively contracting muscle could all result in focal areas of impaired pulsation and lead to the development of cushions which were not related to the orifices of branches. Such an hypothesis also explains the anatomical distribution of these structures. Two factors are required for their presence: (1) marked pulsation of the artery and (2) focal points of fixation. It is, therefore, not surprising to find that they are prominent in the coronary arteries which lie on the surface of the heart and are tethered by the muscular branches penetrating the myocardium. Similarly, both the lower brachial and popliteal vessels are unrestricted because they lie in the popliteal and antecubital fossae and yet are fixed by the geniculate arteries and the vessels given off at the elbow joint respectively.

An interesting feature in the distribution of these muscular cushions is the observation that they are much more prominent in the lower than in the upper limbs. That gravitational forces can have played no part in this predilection is evident since the majority of the foetuses must have spent their existence in an inverted position. Hamilton and Dow (1939) have found that in the dog, where also gravity can play little part, the systolic pressure was higher, and, what is probably more important, the pulse pressure curve was much steeper in the abdominal than in the thoracic aorta. This finding suggests that the difference in the pulse wave in the lower limbs is a function, not of gravity, but of the action of the aorta, a conclusion which correlates well with the present findings. It would seem probable that it is the

steepness of the pulse wave rather than its height which is the main factor in dictating the presence or absence of cushions. The steeper the pulse wave, the more sudden and damaging would be its effects at branchings. For this reason the absence of cushions in the tibial and radial vessels is also not unexpected, for when the pulse wave reaches these arteries it is flattened and much less steep than in the larger trunks (Sodeman, 1956).

In the present series of cases muscular cushions were not a feature of either the femoral or upper part of the brachial artery. It would seem probable that in the case of the femoral vessel this is due to the close support given to it by the muscles forming the subsartorial canal. This would uniformly damp down the pulsation around the whole of its circumference so that the stresses produced at branchings would be mitigated. The more rounded pulse wave in the arm and also the lack of any large branches to fix the upper part of the brachial artery probably dictate the absence of cushions in this vessel.

The aorta presents an apparent discrepancy in the argument put forward above, for it is in this vessel that pulsation is most marked and yet, paradoxically, cushion formation is not prominent. This, however, is also in accordance with the hypothesis, for it holds that the *raison d'être* of these structures is the inability of the circular muscle of the peripheral arteries adequately to resist a longitudinal stress. In contrast to the peripheral vessels, the main strength of the aorta lies in the presence of its many concentric elastic membranes. These membranes could resist a stretching force just as well as a dilating one, so that there is little need for the development of muscular cushions. Nevertheless, even in this vessel there is a tendency for the smooth muscle at the margins of branches to become longitudinally orientated.

No measurements of the relative thickness of the intima in males and females were attempted in the present study. However, Dock's finding that the male intima was thicker than the female, and his suggestion that this was due to a higher stroke volume of the male heart, is quite compatible with the hypothesis put forward above.

The manner by which the cushions are formed and the significance of the elastic tissue reduplication is also of great interest. A close relationship between muscle and elastic tissue changes in arterial walls was observed by Jores (1903, quoted by McMeans, 1915). Jores believed that splitting of elastic tissue is brought about by a mechanical forcing apart of the fibres due to an overgrowth of smooth muscle cells and that a muscle cell hyperplasia always precedes the splitting of elastic fibres. That muscle cells can migrate from the media into the intima through gaps in the internal elastica was shown by Gross, Epstein, and Kugel (1934) in their study of adult coronary arteries. More recently Altschul (1950) also described smooth muscle migration from the media into the intima in experimental and human arteriosclerosis and observed that these migrating cells were radially orientated.

Some support is given to these observations by the present study. The primary role of the media in the formation of intimal cushions has been stressed and the radial rotation of medial muscle fibres both in the formation of longitudinal muscle bands and of fibres which are apparently traversing fenestrae in the internal elastica has been described. Even in the earliest stages of splitting of this membrane evidence of muscles migrating can be found (Fig. 6), suggesting a causal relationship between the two events. On this basis, too, an interpretation can be made of the sequence of events in the formation of a cushion. The primary factor is the pulsatile stress which stimulates a local migration and longitudinal reorientation of the circular muscle of the media with the formation of a simple longitudinal band. If the stress is marked, then there must also be a thickening or hypertrophy of the wall at this point. This is accomplished by a migration of muscle through the internal elastic lamina which is accompanied by a splitting of this membrane and, if sufficiently marked, results in a gross musculo-elastic thickening of the intima (Figs. 3 and 4). The character of the splitting of the internal elastica, whether coarse or fibrillary, depends perhaps on the rate of the process, mild stresses with gradual hypertrophy resulting in an indolent and coarse splitting of the lamina, and severe ones with a rapid intimal thickening and a fine fibrillary mesh.

When the development of these cushions is viewed in this light it is clear that the elastic tissue changes associated with them are not degenerative in nature but are part of a remodelling process. It is difficult to be certain of the significance of the fibrous tissue which is prominent in some cushions. In this connexion the work of Burton *et al.* (1955) is of interest. These workers have suggested that elastin is a derivative of collagen and showed that, by suitable treatment, collagen can be converted *in vitro* into a material which is virtually identical with elastin. If such a close relationship does exist, then it would hardly be surprising that, especially in those cushions with a fine elastic mesh and a relatively quick forma-

tion of elastic fibrils, there should sometimes be an appreciable amount of connective tissue.

The significance of the occasional deposits of lipoid is also uncertain. Its presence was not related to the presence or absence of fibrous tissue. This feature was also noted by Moon. It seems probable that it is but a manifestation of local stress and is akin to the fatty flecking which is seen so commonly around the branches of the aorta and in the muscular arteries of children.

I wish to thank Professor J. H. Biggart for his help and encouragement during the course of this work. Mr. D. Mehaffey was responsible for the photographs.

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THE IDENTIFICATION OF A FLUORESCENT REDUCING SUBSTANCE IN THE URINE OF PATIENTS WITH RHEUMATOID ARTHRITIS

THE EXCRETION OF 3-HYDROXYANTHRANILIC ACID IN THIS AND OTHER CONDITIONS

BY

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The report that 2,5-dihydroxyphenylpyruvic acid occurs in the urine of patients with collagen disease has not been confirmed, and no evidence was found for regarding collagen disease as being due to an inherent error in tyrosine metabolism as suggested by Japanese workers.

A strongly reducing substance was conspicuous in the urine of patients with rheumatoid arthritis but not in that of normal persons. This substance was identified as 3-hydroxyanthranilic acid (3-HAA).

A method is described for the measurement of 3-HAA in urine, employing ether extraction, paper chromatography in a formate solvent, and visual assessment by fluorescence in ultra-violet light and colour with Ehrlich's reagent.

Normal persons excreted from less than 125 to 375 μg./day (average rather lower than 200 μg./day); those suffering from rheumatoid arthritis excreted from less than 125 to 2,250 μg./day (average 820 μg./day).

The excessive excretion of 3-HAA in persons with rheumatoid arthritis was investigated in terms of other aspects of the activity of the disease. Some relationship was suggested between excretion levels, the erythrocyte sedimentation rate, and the sensitized sheep cell test.

Cases of osteoarthritis, other orthopaedic conditions, and miscellaneous diseases were examined. A few of the latter, including diseases of the haemopoietic system, were associated with increased excretion of 3-HAA.

It has recently been claimed that in the urine of patients suffering from one or other of the collagen diseases there may be detected a postulated but hitherto unisolated intermediate of tyrosine metabolism, namely, 2,5-dihydroxyphenylpyruvic acid (Nishimura, Shibata, Yasui, and Okamoto, 1956). This substance was recognized by its purple fluorescence in ultra-violet light after paper chromatography. It was characterized by reduction of ammoniacal silver nitrate and Brigg's reagents and by reactions with ferric chloride, Fenton's and hydrazine reagents. Its chromatographic behaviour in two solvent systems and its ultra-violet absorption spectrum were found to be the same as those of a synthetic specimen of 2,5-dihydroxyphenylpyruvic acid. In the system n-butanol:acetic acid:water (4:1:1) two spots were obtained, depending on whether the urine was acid or neutral during its concentration; one of the spots was thought to be the quinone derivative of 2,5-dihydroxyphenylpyruvic acid and was seen also in chromatograms of the synthetic substance.

In 38 cases of collagen disease the urine contained the substance described. It was absent from the urine of normal persons, those suffering from a variety of other diseases, or unaffected relatives of patients with collagen disease. The authors concluded that a fundamental biochemical clue to the aetiology of collagen diseases had been found, and that these diseases must be grouped with phenylketonuria, alcaptonuria, tyrosinosis, and albinism in that they are caused by an inherent error in the metabolism of tyrosine.

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Examination of Urine for 2,5-Dihydroxyphenylpyruvic Acid

The Japanese workers' description of the preparation of urine extracts for chromatography was limited to the following paragraph:

"Either as it was or acidified with 10% H_2SO_4, the 24-hr. urine of diffuse collagen disease patients was concentrated till about 20 ml. of it remained, and after another further treatment of it with 60% alcohol, and the resultant elimination of inorganic salts, the urine thus obtained was subjected to experiment. Then one-dimensional paper chromatography was performed."

In an attempt to follow the above procedure 250 ml. of urine (freshly collected in acid to give a final *p*H of 3) from a case of acute rheumatoid arthritis was distilled at reduced pressure under nitrogen, the external temperature being kept below 40° C. until a few millilitres of viscous residue remained. This was taken up in alcohol to a concentration of 60% and centrifuged. The supernatant was subjected to chromatography overnight on Whatman No. 1 paper by the ascending technique using the solvent system specified by the Japanese workers (n-butanol:acetic acid:water 4:1:1). When viewed under ultra-violet light ("hanovia" medium pressure quartz arc with Wood's glass filter or "hanovia" chromatolite Hg resonance lamp 2537Å) a large number of fluorescent areas was detectable and subsequent spraying with ammoniacal silver nitrate solution (0.1 N $AgNO_3$ 25 ml., ammonia S.G. 0.88 8 ml., water to 50 ml.) revealed a number of reducing substances. However, no fluorescent reducing substance was seen which had the R_F value stipulated by Nishimura and his colleagues. Furthermore, and perhaps of especial importance in view of the number of closely related compounds into which the quinol sought might be transformed, no difference was detectable in urine from normal individuals and rheumatoid patients when chromatography was carried out in the butanol system or in phenol:water:H_2SO_3, a solvent more suitable than the former for the preservation of a highly oxidizable substance (McMillan, 1957).

An attempt was made to purify the urine before chromatography by adsorption upon alumina (McMillan, 1957). A readily available quinol derivative fairly closely related to that being sought, namely, 2,5-dihydroxybenzoic acid (gentisic acid), was added to whole urine in amounts of 1 μg./ml. The quinol remained in the filtrate during alumina treatment whereas under identical conditions the compound 3,4-dihydroxyphenylacetic acid was adsorbed and none was detected in the filtrate. In neither the eluate nor the filtrate of urine from a case of active rheumatoid arthritis could a fluorescent reducing substance be found to correspond with the Japanese description.

When fresh urine, collected in acid, was extracted with ether at *p*H 3 and the ether-soluble portion chromatographed, a fluorescent reducing substance was conspicuous in the rheumatoid specimens but not in those from normal persons. The ether extracts were distilled to dryness at reduced pressure at a temperature not exceeding 40° C., and the residue, which was derived from 250 ml. urine, was taken up in 1 ml. ethanol. Upon chromatography, 0.02 ml. of this solution gave an easily visible purple spot when viewed under ultra-violet light and had an R_F of 0.84 in the butanol system and 0.61 in the phenol system. The former R_F is higher than either of the values quoted by the Japanese workers for 2,5-dihydroxyphenylpyruvic acid or its quinone.

Identification of the Fluorescent Reducing Substance in Urine from Rheumatoid Patients as 3-Hydroxyanthranilic Acid

The extract described above was chromatographed in the butanol system and the paper was sprayed with a variety of colour-producing reagents with the following results at the site of fluorescence:

Ammoniacal Silver Nitrate. — Dark grey; reduction occurred in the cold and preceded that of any other substance present elsewhere on the paper.

Gibbs' Reagent (2,6-Dichlorquinone Chloroimide 0.2% w/v in Ethanol).—(1) Reagent alone, a purple colour exclusive to the fluorescent spot developed slowly. (2) The reagent diluted with an equal volume of 0.66 M phosphate buffer at *p*H 7.0 gave an immediate purple colour with various colours appearing later in neighbouring areas. (3) The reagent followed by saturated sodium bicarbonate solution or borate solution gave an immediate blue colour at the fluorescent spot, quickly followed, especially in the case of borate, by an array of colours obscuring the original single spot.

Ferric Chloride (0.2% w/v in Water).—A faint yellow colour appeared.

Potassium Ferricyanide (6.6% Aqueous Solution in Phosphate Buffer at *p*H 5.9).—This gave a pink colour.

Potassium Iodate (1% w/v in Water).—This gave a grey colour.

Fenton's Reagent (Nishimura, Yasui, Okamoto, Kanazawa, Kotake, and Shibata, 1958).—This gave a brown colour.

Ehrlich's Reagent.—This is made up of *p*-dimethylaminobenzaldehyde, 10% w/v, in concentrated HCl solution, 1 vol., acetone, 4 vol., mixed immediately before use, and gave a slowly developing distinct orange-yellow colour.

The following reagents either gave no colours at all in the appropriate area or else gave colours which did not properly coincide with the fluorescent spot: Diazotized sulphanilic acid, p-nitraniline

and p-anisidine respectively, ninhydrin, nitroso-naphthol reagent, xanthydrol reagent, Altman's reagent, 2,4-dinitrophenylhydrazine (for all the preceding reagents see Smith, 1958), Millon's reagent, Brigg's reagent (Nishimura *et al.*, 1958), preceding reagents (see Smith, 1958), Millon's Roux reagent (Roux, 1951) and bromothymol blue-acidified potassium iodide-starch ammonia (as used for homogentisic acid by Consden, Forbes, Glynn, and Stanier, 1952). The fluorescent substance was seen to develop a brown colour when the papers were exposed to the atmosphere for an hour or two ; it was unstable in alkaline solution, a *p*H of 9 causing appreciable loss.

By consideration of the above properties and by comparing the observed R_F in various solvent systems with those published for phenolic acids known to occur in urine (for references see Smith, 1958), it appeared probable that the fluorescent substance was 3-hydroxyanthranilic acid. A specimen of this substance (Roche) proved to have the same fluorescence under both ultra-violet light sources and to give the same colour reactions as the unknown substance. When added to the unknown substance in a urine concentrate it had an identical R_F in four solvent systems, namely, 0.61 in phenol : water : H_2SO_3 ; 0.84 in n-butanol : acetic acid : water (4 : 1 : 1), 0.52 in benzene : acetic acid : water (125 : 72 : 3), and 0.63 in sodium formate : formic acid : water (10 g. : 1 ml. : 200 ml.). Both substances were largely destroyed in the system iso-propanol : 0.88 ammonia : water (200 : 10 : 20).

Semiquantitative Measurement of the Amount of 3-Hydroxyanthranilic Acid in Urine

The fluorescence of 3-hydroxyanthranilic acid (3-HAA) in ultra-violet light could be detected easily on the chromatogram paper in amounts as low as 0.25 μg., whereas none of the colour reactions was as sensitive as this. Chromatography in the butanol system was unsuited to quantitative work because very many phenolic acids have a similar high R_F value ; urine extracts prepared by simple ether extraction gave neighbouring spots which were highly fluorescent under ultra-violet light, and, though they could be distinguished from 3-hydroxyanthranilic acid by their blue colour, they partially, or sometimes completely, obscured the purple colour of 3-HAA. The benzene system was unsuitable because the spots became too spread out. Both the phenol and formate systems gave sharply defined spots and some urine extracts could be satisfactorily assessed in terms of standard amounts of 3-HAA run on the same sheets of paper. Many, however, showed one or more fluorescent substance which interfered more or less seriously with such a comparison. Two-dimensional chromatography, employing the phenol system followed by the formate, failed to eradicate the interference. After the use of any of the above solvent systems colour development on the paper with Ehrlich's reagent or buffered potassium ferricyanide solution proved insufficiently sensitive and ammoniacal silver nitrate or Gibbs reagent at various *p*H values insufficiently specific.

Owing to the presence of a basic group, 3-HAA is not extracted by ether from highly acid aqueous solution. When urine was acidified with one-tenth of its volume of concentrated HCl and extracted with ether, the concentrated ether extracts on chromatography usually contained much material fluorescent in ultra-violet light, whereas subsequent extraction of the aqueous residue after adjustment of the *p*H to 3 gave a preparation which frequently showed only one fluorescent area, that due to 3-HAA. These findings were applicable to chromatography in the phenol, formate, and butanol systems. At each *p*H ether was extracted with four equal portions whose total volume equalled that of the aqueous phase. Experiments showed that, when added to the urine in amounts as high as 1 μg./ml., 3-HAA could be satisfactorily accounted for during the processes of extraction, concentration, and chromatography ; the four combined extracts made at *p*H 3 contained as far as could be assessed by visual comparison the whole of the added 3-HAA, whereas the four ether extracts of the highly acid urine and two additional extracts made at *p*H 3 subsequent to the four normally prepared showed no trace of 3-HAA.

After such a procedure of double ether extraction some urine extracts continued to give additional fluorescent spots. Commonly there was found a blue spot immediately above the purple 3-HAA in both phenol and formate systems ; the fluorescence of this substance was much greater under the "hanovia" 2537 Å lamp than under the Woods glass whereas that of 3-HAA was similar under both, so that there was no danger of confusion. In order to guard against the possibility of interference at the actual site of 3-HAA, though this appeared unlikely, after assessment under ultra-violet light the chromatogram was always sprayed with Ehrlich's reagent and some hours later the intensities and sizes of the yellow spots were compared with those of the standards. Amounts less than 0.5 μg usually did not show up, but for amounts greater

than this there was never any great discrepancy between the values obtained by the two methods of comparison. Chromatograms developed in the phenol system were easily assessed by fluorescence in ultra-violet light and Ehrlich colour (pink), the paper being freed of phenol by benzene extraction followed by hanging in a current of warm air for two to three hours. In fact, in 80% of the assays to be reported duplicate chromatograms were thus run; there was always close agreement with the standard method using formate. After double ether extraction some chromatograms run in the butanol system and dried in warm air were sprayed with Gibbs' reagent followed by saturated sodium dihydrogen phosphate solution. This method of assessment was quite unsatisfactory as the initial spots of 3-HAA were rapidly obscured by several other closely situated substances (cf. Tompsett, 1959).

It was found that urine acidified to *p*H 3 or thereabouts maintained its content of 3-HAA for many days when stored in the refrigerator. The ether extracts were unstable and were always assayed as soon as possible, usually on the day of preparation. Similarly the developed chromatograms were always assessed as soon as the papers were dry.

The possibility of completing the assay of 3-HAA by spectrofluorometric measurement was not investigated in this study.

Method

Preparation of Extract.—Urine was collected for 24 hours, 15 ml. of 10% H_2SO_4 being used as preservative. To a filtered aliquot measuring 10% of the whole specimen was added one-tenth of its volume of concentrated HCl. The mixture was then shaken for two-minute periods with an equal volume of peroxide-free ether divided into four equal portions. Centrifugation was usually necessary to effect separation. After each extraction the ether layer was pipetted off and discarded. The *p*H of the aqueous phase was then adjusted with a volume of 10 N NaOH equivalent to that of the concentrated HCl previously used, followed by more dilute alkali until a *p*H of 3.5 was reached (B.D.H. 2540 indicator paper). Ether extraction was repeated with the same amounts as before. The four resulting ether fractions were combined and distilled to dryness at reduced pressure under nitrogen at a bath temperature not exceeding 40° C. The residue was taken up in four small successive amounts of ethanol and the solution was evaporated to a volume of 0.5 ml. This pale, straw-coloured solution was used for chromatography.

The general chromatographic technique was that described by McMillan (1957). The paper was marked out into four strips (each 2 cm. wide) for the urine extract (usually 0.01, 0.005, 0.0025, and 0.00125 ml.) and four for the standard 3-hydroxyanthranilic acid solution (2.0, 1.0, 0.5, and 0.25 μg.). Before use the standard was diluted 1 in 5 in ethanol from a stock refrigerated solution containing 20 mg./20 ml. ethanol to which 0.05 ml. of concentrated HCl had been added. The solvent (sodium formate 10 g., water 200 ml., formic acid 1 ml.) was renewed on each occasion. Two or three sheets of paper were hung in the same tank for two to three hours until the solvent front reached about 25 cm. A sheet of sponge rubber and a wrapper of cellophane paper were applied to the underside of the lid of the tank which was then quickly sealed by pressure from above (F. Albert-Recht, personal communication). The sheets were dried in the air at room temperature using a hair dryer. The amount of 3-hydroxyanthranilic acid in each of the four urine extract chromatograms was assessed visually by transmitted light from both the ultra-violet lamps previously described. The paper was then sprayed with Ehrlich's reagent and further comparisons made after about 18 hours.

Results

The excretion of 3-hydroxyanthranilic acid by 83 persons was determined. Twenty-five patients suffering from rheumatoid arthritis (all of whom were in-patients of the Rheumatic Unit, Northern General Hospital, Edinburgh) excreted quantities ranging from less than 125 μg. (the minimal amount detectable by the method) to 2,250 μg./day, the average figure being 820 μg./day. In a few instances excretion was measured on more than one occasion, when values of the same order were found. The figures bore no relationship to the age or sex of the patient. The patients had received no steroid therapy or other drugs for at least three days before the urine was collected.

Twelve healthy laboratory workers excreted from less than 125 to 375 μg./day, the average being rather lower than 200 μg./day. Fig. 1 shows that whereas only two of these normal persons excreted more than 250 μg./day, all rheumatoid subjects save three excreted 375 μg. or more/day. There would therefore appear to be some considerable difference between these two groups. One normal person who received heavy doses of either aspirin or panadol for three days showed the same low excretion three days later.

Analysis of the group of rheumatoid patients failed to show any correlation between the level of excretion of 3-hydroxyanthranilic acid and some of the criteria which might be used to assess the severity of the disease, namely, the duration of symptoms, the extent of tissue damage as shown radiologically, or the degree of anaemia. Comparison of the erythrocyte sedimentation rate (E.S.R.) measured by the Westergren method with excretion of 3-hydroxyanthranilic acid

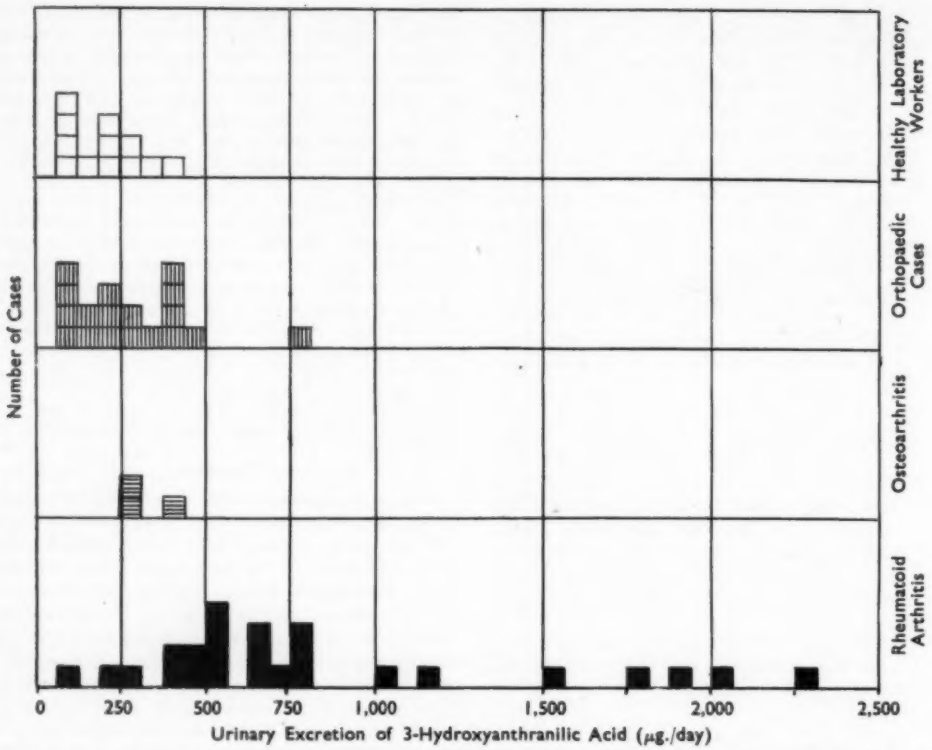

FIG. 1.—Urinary excretion of 3-hydroxyanthranilic acid in rheumatoid arthritis, osteoarthritis, and orthopaedic conditions as compared with normal persons.

showed that the cases fell into two groups (Fig. 2). The first comprises the seven highest excretors, that is, all who excreted 1,000 μg. or more per day, who had E.S.R. values ranging from 27 to 86 mm./hour (average 61 mm./hr.). The E.S.R. of the remaining 18 cases ranged from 3 to 54 (average 32) mm./hr. and in these the 3-hydroxyanthranilic acid excretion never exceeded 750 μg./day. There is thus a trend towards proportionality between the two values. Comparison with the titres obtained in the sensitized sheep cell test (for details see Duthie, Brown, Knox, and Thompson, 1957) showed a similar pattern of distribution as obtained for the E.S.R. for the seven highest excretors, all of whose titres were positive, ranging from 1 : 128 to 1 : 2,048 (Fig. 3). The remaining 18 cases, however, had titres from 1 : 8 to 1 : 2,048. Thus all patients with a high excretion of 3-hydroxyanthranilic acid gave a positive sensitized sheep cell test, but a positive titre was not necessarily accompanied by a high level of excretion. Degrees of rheumatoid activity have been classified by Duthie *et al.* (1957) in terms of E.S.R., haemoglobin levels, joint involvement, and systemic disturbance. The activity of the disease of some of the above patients was independently assessed by the former workers. Three cases were allocated to group 1 (inactive), 18 to group 2 (moderately active), and none to group 3 (very active). No relationship with the level of excretion of 3-hydroxyanthranilic acid could be discerned. Similarly, by the same authors' classification of functional capacity, one case fell in group 1 (no disability), 14 in group 2 (moderate incapacity), three in group 3 (marked incapacity), and one in group 4 (confined to bed or chair); again no correlation with excretion could be found.

A small group of patients with osteoarthritis was investigated. These patients were receiving similar basic treatment and diet in the same wards and at the same time as the rheumatoid group.

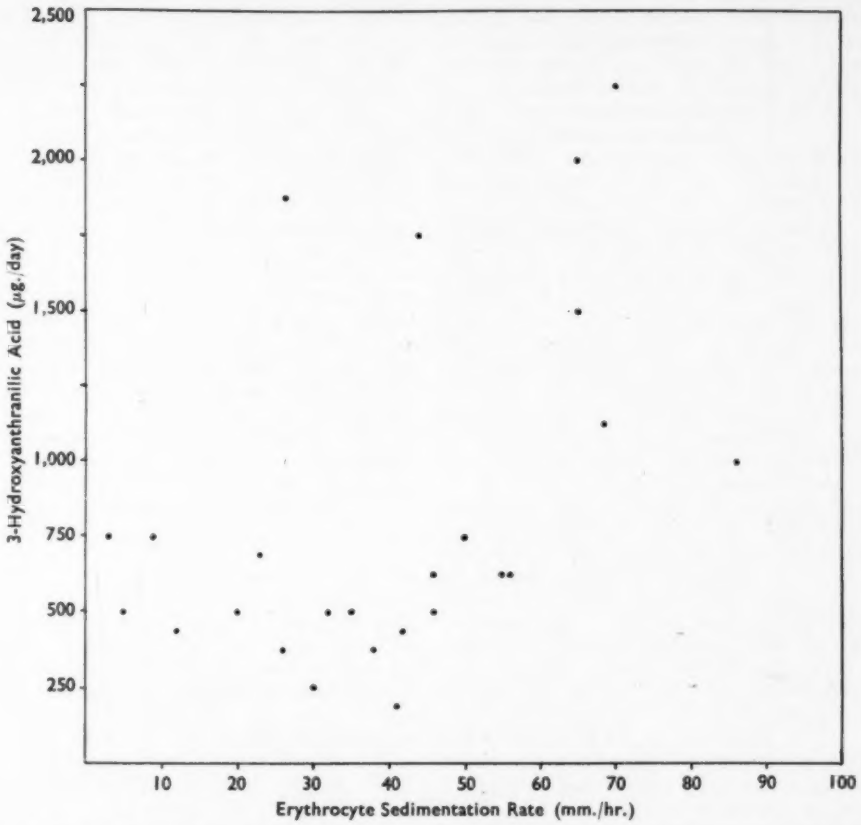

FIG. 2.—The relationship between urinary excretion of 3-hydroxyanthranilic acid and the erythrocyte sedimentation rate in patients suffering from rheumatoid arthritis.

but 3-hydroxyanthranilic acid excretion showed values not in excess of those of normal persons (Fig. 1). A larger group of 18 orthopaedic cases was studied. Of these patients, some were ambulant at the time of examination, some were more or less immobilized. Fig. 1 shows that in these cases the level of excretion of 3-hydroxyanthranilic acid was similar to that of normal persons, the average being 240 μg./day; with a single exception the range extended from less than 125 to 440 μg./day. A wide range of traumatic and non-traumatic conditions was represented; there was no obvious relationship between excretion levels and such factors as extent of tissue injury or degree of immobilization. The exceptional patient who excreted as much as 750 μg./day was one who 15 weeks previously had undergone fusion of the hip on account of an old injury.

In a miscellaneous group (Fig. 4), one case of ankylosing spondylitis showed a low excretion, as did a single case of disseminated lupus erythematosus receiving prednisolone. In two cases of psoriatic arthritis the excretion was low in one and very high in the other (1,750 μg./day). Two patients who had undergone partial gastrectomy two days before the collection of urine showed a low excretion.

In medical conditions such as myocardial infarction, pulmonary infarction, chronic nephritis, uraemia, hepatic cirrhosis, duodenal ulcer, etc., excretion figures were uniformly low. This was the rule also with patients having sepsis or anaemia, including cases of staphylococcal

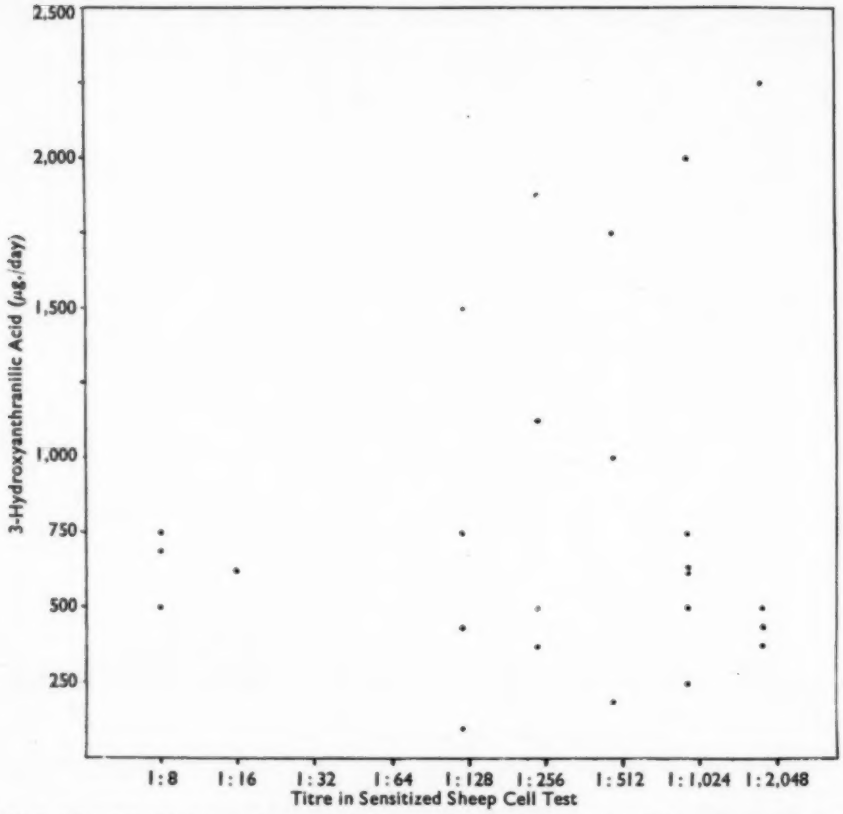

FIG. 3.—The relationship between the titre obtained in the sensitized sheep cell test and the urinary excretion of 3-hydroxyanthranilic acid.

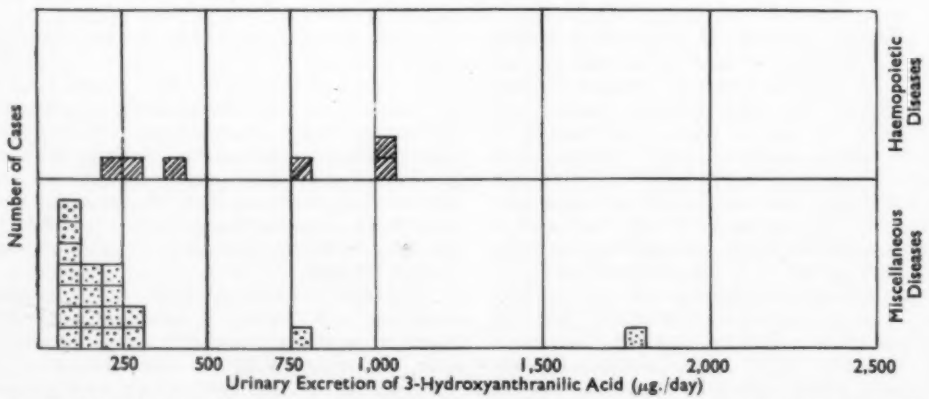

FIG. 4.—Urinary excretion of 3-hydroxyanthranilic acid in diseases of the haemopoietic system and in other miscellaneous conditions.

pneumonia and active pulmonary tuberculosis. Fever alone caused no rise. In view of the possible relationship between the E.S.R. and the excretion of 3-hydroxyanthranilic acid in rheumatoid arthritis special attention was paid to patients having a very high E.S.R. from other causes. All gave low figures with the exception of one patient with myelosclerosis with aplastic anaemia who had an E.S.R. of 125 mm./hour and an excretion of 3-hydroxyanthranilic acid as high as 1,000 μg./day. The other five patients having blood diseases included in this survey also showed a tendency to raised outputs. Thus three patients with chronic myeloid leukaemia, whose white cell counts were 209,000, 182,000, and 60,800/c.mm. respectively, excreted 250, 750, and 1,000 μg./day ; the second of these had undergone no treatment, the other two were receiving irradiation therapy. Two other patients suffering from megakaryocytic leukaemia and myelomatosis, whose white cell counts were not raised, excreted 190 and 440 μg./day respectively.

Discussion

This study fails to substantiate the claim of Nishimura *et al.* that 2,5-dihydroxyphenylpyruvic acid is excreted in the urine by patients with rheumatoid arthritis. The data published by the Japanese workers were incomplete, but an approximation to their method of identification failed to demonstrate any such compound. In the absence of excretion of 2,5-dihydroxyphenylpyruvic acid there is no evidence for the hypothesis that the collagen diseases are due to an inherent error in the metabolism of tyrosine.

Chromatographic examination of the urine in cases of rheumatoid arthritis for a strongly reducing substance has shown, however, that free 3-hydroxyanthranilic acid is excreted in increased amount as compared with normal subjects. The compound is known to be a normal metabolite of tryptophan ; it is the immediate degradation product of 3-hydroxykynurenine and a precursor of nicotinic acid. It has so far been of limited medical interest, mainly as a possible carcinogen. Since the suggestion that o-aminophenols were carcinogenic (Bonser, Clayson, Jull, and Pyrah, 1954), attention has been drawn to the two naturally occurring compounds, namely, 3-hydroxykynurenine and 3-hydroxyanthranilic acid, which have this structure. In fact, 3-hydroxyanthranilic acid was shown to produce cancer of the bladder in mice (Watson, 1956) and to be excreted in the unconjugated state in increased amount in human cancer of the bladder (Boyland and Williams, 1956). The latter authors, who used a method involving adsorption on charcoal and gradient-elution chromatography, give the normal excretion of free 3-hydroxyanthranilic acid as 8 to 32 (average 20) mg./10 l. urine ; this is considerably higher than the values reported in the present paper.

Dalgliesh and Tekman (1954) reported an increased urinary output of kynurenine and 3-hydroxykynurenine in half of a series of febrile patients, suggesting it was due to increased tissue breakdown ; loss of weight without fever and thyrotoxicosis did not have this effect. Musajo, Benassi, and Parpajola (1956) reported that recession of fever did not arrest the excessive excretion of kynurenines. In a large series, the same authors (1955), using paper chromatography, found "very small amounts" of 3-HAA to be excreted by normal human subjects. In 11 out of 13 subjects with pulmonary tuberculosis excretion was definitely higher. Also in 59 out of 76 cases of leukaemia of various types, myelomatosis, Hodgkin's disease, and lymphosarcoma, considerable quantities of kynurenine and 3-hydroxykynurenine were found, but none was detectable in normal persons ; no details were given in regard to 3-HAA, apart from a statement that excretion was raised in about one-third of the cases. The authors emphasized that the detection of tryptophan metabolites in human urine in the absence of special diets or administration of tryptophan was rare, and such excretion was linked with pathological conditions.

An increased excretion of 3-HAA in any of the collagen diseases has not been reported previously. In the present series of cases the magnitude of the increase would appear to bear no relation to any of the major features of the disease except perhaps the E.S.R., where there was some proportionality, and the sensitized sheep cell test, which was always positive in association with the highest excretion rates. A high E.S.R. *per se* does not lead to increased excretion since many non-rheumatoid patients had a very high E.S.R. without such an increase. The rheumatoid patients had received no steroid or other drugs for at least three days before 3-HAA excretion was measured. A normal person who received heavy doses of either aspirin or panadol for three days showed no increase in 3-HAA excretion three days later. The effect of drugs on excretion by the rheumatoid patients was not investigated.

In agreement with Musajo *et al.* (1956), no direct relationship was found between 3-HAA excretion and fever ; but contrary to the above report no increase was found in active pulmonary tuberculosis.

The raised excretion in some patients with haematological disorders is of interest and merits a further survey and investigation. Cases of erythrogenesis imperfecta have been reported to excrete abnormal amounts of anthranilic acid (Altman and Miller, 1953). In diseases of the blood-forming organs, then, it would appear that more than one tryptophan metabolite may be excreted in excess. Whether metabolites other than 3-HAA are involved in rheumatoid arthritis has not been determined and there is no evidence to suggest that the findings in rheumatoid arthritis are in any way related to those in blood disease.

An increase in urinary 3-HAA could be due to increased tryptophan catabolism. However, many patients in whom tissue was probably wasting failed to show this effect and in rheumatoid arthritis itself there was no correlation with such wasting. Possibly 3-hydroxyanthranilic acid could accumulate as a result of enzymic imbalance such as can be produced *in vivo* in the case of certain other tryptophan metabolites by vitamin deficiency. Further insight into the abnormality in rheumatoid arthritis might be gained by studies along these lines.

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FACTORS AFFECTING THE UPTAKE OF LISSAMINE GREEN BY SERUM PROTEINS*

BY

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Eight physicochemical factors which affect the uptake of lissamine green on filter paper impregnated with serum proteins have been examined, and their relevance to the staining of electrophoretically separated protein fractions is discussed. It is shown that grade of paper, weight of protein applied, separate and combined denaturation and staining time, temperature and concentration of staining solution, concentration of denaturant, and type of protein all influence the weight of dye absorbed per unit weight of applied protein, and must be rigidly standardized if valid quantitative results are to be obtained.

Five sets of conditions are obtained for optimal staining and it is found that separation of denaturant from dye yields the best procedure. It is concluded that lissamine green is an excellent dye for the staining and quantitative estimation of separated protein fractions in paper electrophoresis, and that conditions can usually be arranged to produce a linear relation between dye uptake and protein concentration in an experimentally efficient manner.

Despite the large number of publications dealing with the separation and staining of serum proteins by paper electrophoresis, there is a relative paucity of information concerning the role of physicochemical factors. McDonald (1952, 1955), McDonald, Urbin, and Williamson (1951), McDonald, Lappe, Marbach, Spitzer, and Urbin (1953), and McDonald, Foresman, and Bermes (1957) have shown how such variables as temperature and voltage affect the mobility, while studies such as those of Franglen and Martin (1954), Jencks, Jetton, and Durrum (1955), Franglen (1956) and Henry, Golub, and Sobel (1957) indicate the complexity of the numerous entities influencing the uptake of dye by the separated components.

One result has been to demonstrate the deficiencies of some dyes and the advantages of others. Thus lissamine green has come to be favoured by many workers (Discombe, Jones, and Winstanley, 1954; Abdel-Wahab and Laurence, 1955; Dangerfield and Smith, 1955; Rideout and Prichard, 1955; Gilliland, Johnston, Stradling, and Abdel-Wahab, 1956; Owen, 1956; Gorringe, 1957; Rappaport, Eichhorn, and Fischl, 1957). Unfortunately, most of the dyes studied under the conditions reported to date suffer from some drawback such as variation of dye uptake with protein fraction. This has led to the use of correction factors in some quarters (Cremer and Tiselius, 1950; Köiw, Wallenius, and Grönwall, 1952); but their adoption has been questioned on the ground that there is no adequate standard for comparison (Owen, 1956).

Accordingly, claims that bromophenol blue (Hardwicke, 1954) and lissamine green (Gorringe, 1957) have the same binding capacity for the different protein fractions merit close consideration. The object of the present study is to examine some physicochemical variables affecting the uptake of lissamine green and thus test its suitability as a stain in paper electrophoresis.

Materials and Methods

Protein Solutions.—Solutions of whole serum, albumin, and γ-globulin were prepared by dilution with 0.9% sodium chloride solution. The electrophoretic analysis of the latter two fractions was very satisfactory. Pooled normal human serum† containing merthiolate as preservative, normal human serum albumin‡, buffered and concentrated, and normal human serum γ-globulin‡ containing merthiolate as additive were used. Stock solutions were standardized both by a biuret copper sulphate method and a micro-

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† By courtesy of the Australian Red Cross Society, Western Australian Division.

‡ Commonwealth Serum Laboratories, Melbourne, Victoria.

Kjeldahl method in which a conversion factor of 6.54 was employed (Sunderman, Sunderman, Falvo, and Kallick, 1958).

Paper.—In general, Whatman No. 3MM filter paper was used throughout this work. To study the effects of other grades, Whatman Nos. 2 and 3 were also tested.

Dye.—Lissamine green S.F. 150* was the sole dye used. Although chromatography reveals the presence of several components, Gorringe (1957) tested seven batches and found the dye to behave similarly in every respect, as did also one batch after repeated use. Its excellent reproducibility has been confirmed in this laboratory.

Eluent.—The elution buffer of *p*H 6.0 recommended by Gorringe (1957) was adopted; it proved to act more rapidly than 40% acetic acid which Owen (1956) used. Solutions of the dye were found to be stable at room temperature for several days.

Absorbance Measurements.—A calibration line of dye concentration versus optical density was constructed with solutions of dye in the elution buffer using a Hilger "uvispek" quartz spectrophotometer and 1 cm. cells. Beer's law was obeyed over the concentration range studied (0–20.5 μg. per ml.) at 635 mμ, the wavelength of maximum absorption. The relation $C = 20.9\ D$ fitted the experimental data to within $\pm 2\%$, where C is the dye concentration in μg. per ml. and D is the optical density.

Staining Process.—To filter paper strips, 10 μl. amounts of protein solution were added from a micropipette so as to form circular areas. The dimensions were found to vary with the concentration. When the spots had dried, the protein was denatured and stained. This was done either simultaneously by immersing for a given time in a solution of dye in 3% aqueous salicylsulphonic acid, or separately by dipping in 3% aqueous salicylsulphonic acid for at least 15 minutes and staining in an aqueous dye bath. This was preferred to heat denaturation in the belief that more control would be gained. Thus Henry *et al.* (1957) proved that "time and temperature of heat-fixation are critical variables" and "their close control is essential for reproducible results." Excess dye was removed with four washings of 2% acetic acid solution (v/v) in water followed by two rinsings in distilled water. The strips then had a perfectly white background and were dried at 30° C.

Dye Uptake Measurements.—Areas of dye spots were calculated by circumscribing 10 times with a planimeter. The spots were then cut out and placed in a measured volume of eluent. Identical areas of background paper provided blanks. When elution was complete, the optical density of each solution was measured and the amount of dye calculated from the calibration equation. All measurements were performed in triplicate and the mean taken.

* Imperial Chemical Industries Ltd., Dyestuffs Division.

Results

The following results describe the effects on lissamine green absorption of (1) grade of filter paper, (2) weight of protein applied, (3) combined denaturation-staining time, (4) concentration of dye, (5) combined denaturation-staining temperature, (6) concentration of salicylsulphonic acid, (7) protein fraction, and (8) separate times of denaturation and staining.

Effect of Grade of Paper.—To test the effect of different types of Whatman filter paper, three 10 μl. volumes of serum protein of concentration 5.22 g. per 100 ml. were pipetted on to each of Nos. 2, 3, and 3MM grade papers. After combined denaturation-staining, the areas of the protein spots were determined by planimetry. They were then eluted and the weights of dye absorbed per square cm. evaluated. The results are shown in Table I.

TABLE I

EFFECT OF GRADE OF FILTER PAPER ON DYE UPTAKE

Grade of Paper	Dye (μg. per cm.^{2})	Area of Spread (cm.^{2})	Thickness	Roughness
2	82·4 ± 0·8	1·062 ± 0·026	+	+
3MM	117·4 ± 2·1	0·647 ± 0·005	++	++
3	161·3 ± 4·6	0·505 ± 0·008	+++	+++

The weight of dye absorbed per unit area when 10 μl. of serum was added to various grades of Whatman filter paper is compared quantitatively with the area of spread and qualitatively with the thickness and roughness. The total serum protein content was 5·22 g. per 100 ml. Numerical values indicate the range of triplicate measurements. The dye concentration was 200 mg. per 100 ml. of 3% aqueous salicylsulphonic acid, the staining time one hour, and the temperature 20° C.

It is apparent that the highest dye uptake is on grade 3 paper, which also gives rise to the smallest protein area. In quantitation by elution, the advantage of a high uptake is somewhat offset by the smaller area and thicker paper to be cut out. Moreover, there is the densitometric disadvantage that No. 3 paper is the roughest and hence the least uniform in surface properties. These factors must be borne in mind when choosing a grade of paper; in the present study a middle course was taken and grade 3MM was selected for use.

Effect of Weight of Protein.—There are two ways of applying quantities of protein to paper. The first is to pipette varying volumes of a fixed concentration of protein solution; the second is to pipette a fixed volume of varying concentrations. The weight of dye absorbed per unit area by both these procedures is shown in Fig. 1. The curves are similar to a concentration of 300 μg. per sq. cm.

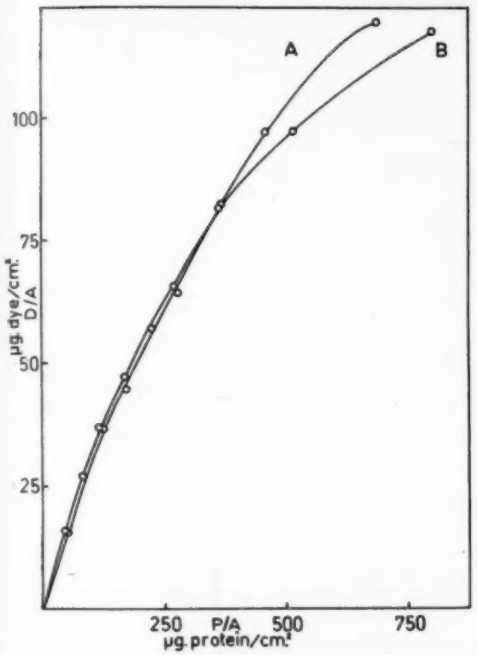

FIG. 1.—Variation of weight of lissamine green absorbed per sq. cm. of paper (D/A) with weight of protein in μg. applied per sq. cm. (P/A). Curve A was obtained by adding various volumes of a fixed protein concentration; curve B was obtained by adding a fixed volume of various protein concentrations. Experimental conditions are described in the text.

In Fig. 2 the ratio of the weight of dye absorbed to the weight of protein applied (D/P) is expressed as a function of (*a*) area of spot, (*b*) volume of solution pipetted, and (*c*) weight of protein applied. The areas and volumes in (*a*) and (*b*), respectively, were those used in drawing the fixed concentration curve A of Fig. 1. The values in Fig. 2(*c*) were those used in drawing the fixed volume curve B of Fig. 1. The conditions employed in obtaining the data of Figs. 1 and 2 were as follows: Dye concentration: 200 mg. per 100 ml. of 3% aqueous salicylsulphonic acid; denaturation-staining time: one hour; temperature: 20° C.

As the protein density rises, the dye molecules find it increasingly difficult to occupy the diminishing number of vacant interstices in the paper. Since P, V, and A are all mutually proportional, the progressive decrease of the dye-protein ratio in each case is to be expected. Similar observations have been made by Gorringe (1957).

Effect of Time.—The ratio of the weight of dye absorbed to the weight of protein applied (D/P) at various protein concentrations under conditions combining denaturation with staining was measured at intervals of 5, 10, 15, 30, 45, 60, 150 minutes, and 24 hours.

From an electrophoretic point of view, the ratio of the weight of dye absorbed to the weight of protein applied (the dye-protein ratio, D/P) is a more significant quantity than the weight of dye absorbed per sq. cm. (the dye uptake, D/A) for the determination of optimal staining conditions. (If elution is preferred to direct densitometry, calculation of the area A is superfluous.) When the dye-protein ratio is identical for all protein concentrations, then the dye uptake is directly related to the weight of protein applied. An even more favourable circumstance occurs when the

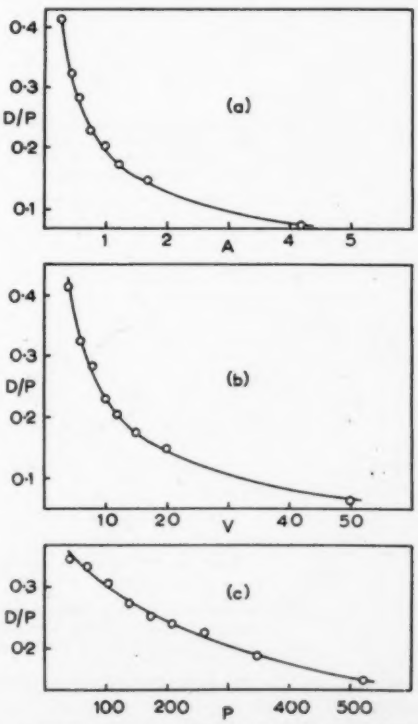

FIG. 2.—Variations of ratio of weight of lissamine green absorbed to the weight of protein applied (D/P) with (*a*) area in sq. cm. (A) covered by various volumes of a 2.09 g. per 100 ml. protein solution, (*b*) volume in μl. (V) of a 2.09 g. per 100 ml. protein solution, and (*c*) weight in μg. (P) of protein applied at a fixed volume of 10 μl. Experimental conditions are described in the text.

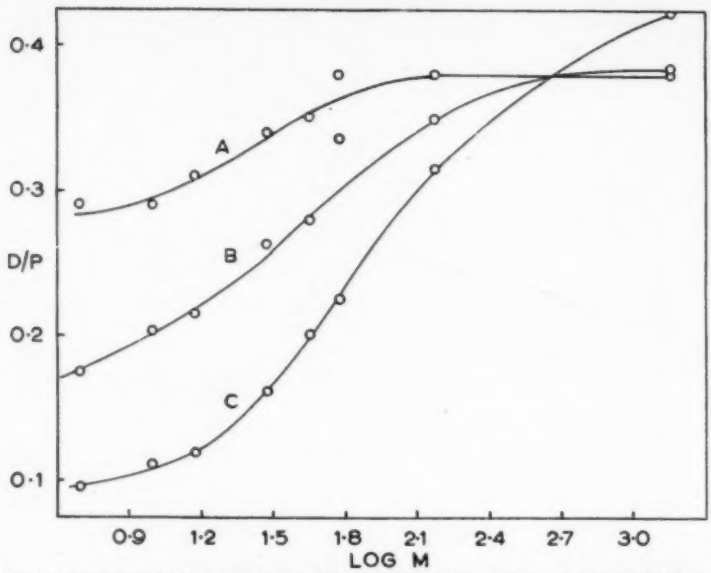

FIG. 3.—Variation of the dye-protein ratio (D/P) with the logarithm of the time in minutes (M) during which combined denaturation-staining took place. Curve A denotes a protein concentration of 0.21 g. per 100 ml.; curve B 1.04 g. per 100 ml.; and curve C 5.22 g. per 100 ml. Experimental conditions are described in the text.

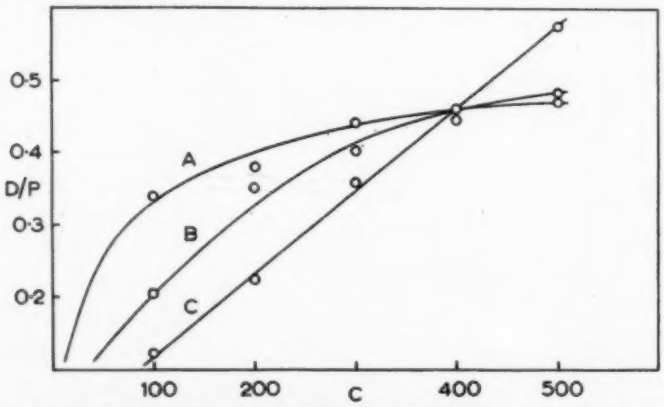

FIG. 4.—Variation of the dye-protein ratio (D/P) with the lissamine green concentration (C) in mg. per 100 ml. at protein concentrations of A: 0·21 g. per 100 ml., B: 1.04 g. per 100 ml., and C: 5.22 g. per 100 ml. Experimental conditions are described in the text.

dye-protein ratio is the same for all serum protein fractions.

Fig. 3 indicates the time-dependence of the dye-protein ratio at three protein concentrations. (The abscissa has been plotted in common logarithms to compress the scale.) The ratio becomes independent of protein concentration at approximately 470 minutes. The dye concentration was 200 mg. per 100 ml of 3% aqueous salicylsulphonic acid, and the temperature 20° C.

Effect of Dye Concentration.—Fig. 4 demonstrates the effect of varying the concentration of lissamine green in mg. per 100 ml. of 3% aqueous salicylsulphonic acid on the dye-protein ratio at three protein concentrations. Denaturation-staining was carried out for one hour at 20° C

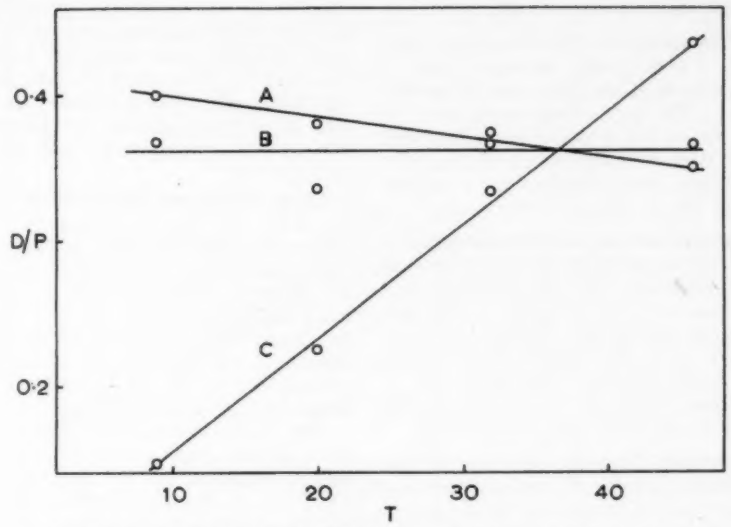

FIG. 5.—Variation of the dye-protein ratio (D/P) with the centigrade temperature (T) at protein concentrations of A: 0.21 g. per 100 ml., B: 1.04 g. per 100 ml., and C: 5.22 g. per 100 ml. Experimental conditions are described in the text.

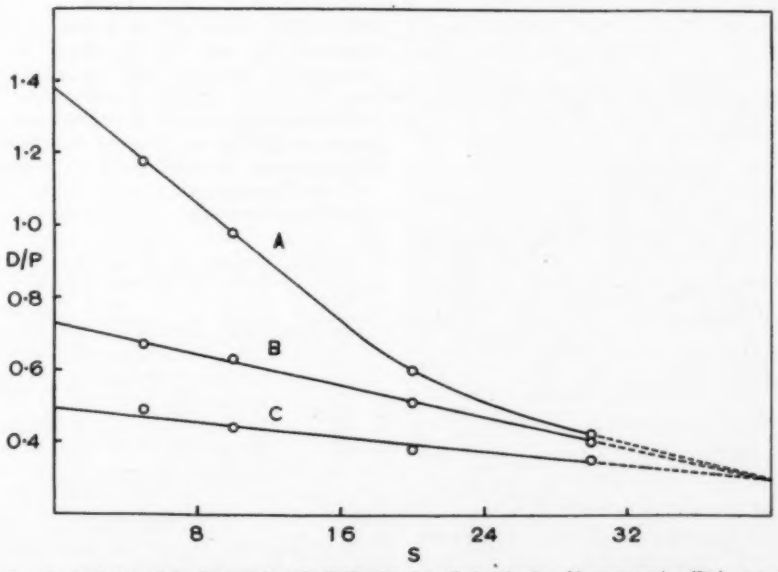

FIG. 6.—Variation of the dye-protein ratio (D/P) with the salicylsulphonic acid concentration (S) in g. per litre at protein concentrations of A: 5.22 g. per 100 ml., B: 1.04 g. per 100 ml., and C: 0.21 g. per 100 ml. Experimental conditions are described in the text.

The point of intersection occurs at a dye concentration of about 410 mg. per 100 ml.

Effect of Temperature.—The dye-protein ratio is seen in Fig. 5 to vary with the temperature of denaturation-staining at the three specified protein concentrations. The dye concentration was 200 mg. per 100 ml. of 3% aqueous salicylsulphonic acid and the time of denaturation-staining was one hour. Under these conditions an optimal temperature of 36° C. was obtained.

Effect of Salicylsulphonic Acid Concentration.—Fig. 6 shows the dependence of the dye-protein ratio on the concentration of salicylsulphonic acid at various protein concentrations. The dye concentration was 200 mg. per 100 ml. of aqueous salicylsulphonic acid, the denaturation-staining time was 24 hours and the temperature 24° C. Extrapolation leads to an approximate value of 40 g. per litre for the optimal acid concentration.

Effect of Protein Fraction.—In view of the preceding results, some of the experimental conditions were altered to gain increased staining efficiency. Using a reduced salicylsulphonic acid content in the combined denaturation-staining solution, the dye uptakes of whole serum, albumin, and γ-globulin were measured. The results are shown in Fig. 7 for which the following conditions pertained: Dye concentration: 200 mg. per 100 ml. of 0.5% aqueous salicylsulphonic acid; denaturation-staining time: one hour; temperature: 21° C.

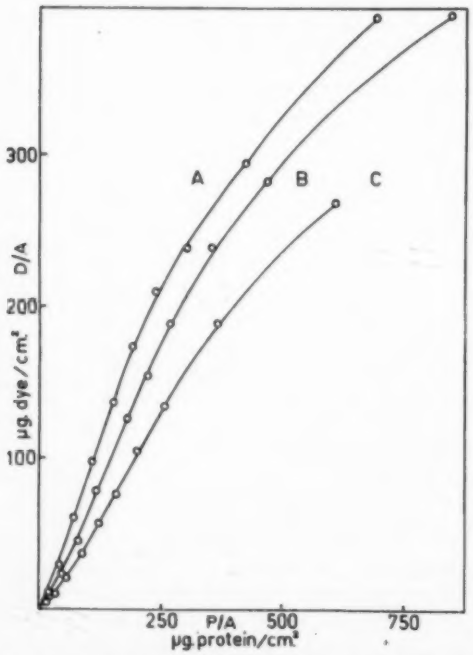

FIG. 7.—Variation of weight of lissamine green absorbed per sq. cm. of paper (D/A) with weight of protein in μg. applied per sq. cm. (P/A) for A: albumin, B: whole serum, and C: γ-globulin. Experimental conditions are described in the text.

Effect of Separate Denaturation and Staining.—In order to obtain quantitative data on the differential effect between combined and separate denaturation and staining on the dye-protein ratio, the following experiment was performed. Before being stained for 15, 30, 45, 60, 150 minutes, and 24 hours, various quantities of protein were denatured on paper in a salicylsulphonic acid solution for 15, 30, 45, 60, 150 minutes, and 24 hours for each staining time.

The results showed that, within experimental error, the dye uptake at a particular staining time was independent of the time of denaturation. Thus denaturation was complete within 15 minutes for the complete range of protein concentrations studied (0.21 to 5.22 g. per 100 ml.) under the following conditions: Dye concentration: 200 mg. per 100 ml. of water; temperature: 17° C.; salicylsulphonic acid concentration: 30 g. per litre. Fig. 8 shows the variation of the dye-protein ratio with the logarithm of the time of staining in minutes after denaturation for 15 minutes. The conditions were those described in this section, and results were obtained at five protein concentrations. The optimal staining time is attained after approximately 23 minutes.

Table II summarizes the five sets of optimal conditions determined in these studies.

TABLE II
SUMMARY OF OPTIMAL STAINING CONDITIONS

Dye Concentration (mg./100 ml.)	Temperature (°C.)	Time (min.)	Salicylsulphonic Acid Concentration (g./litre)	Separate or Combined Staining	Dye-Protein Ratio at Optimal Condition
410*	20	60	30	Combined	0·46
200	36*	60	30	,,	0·36
200	17	23*	30	Separate	0·35
200	20	470*	30	Combined	0·38
200	24	1,440	40*	,,	0·30

* Experimentally determined value.

Discussion

It is clear that, under the conditions described, linearity of dye uptake with protein concentration has not been obtained. Further, the uptakes of normal human serum, albumin, and γ-globulin differ appreciably, the order of increasing uptake

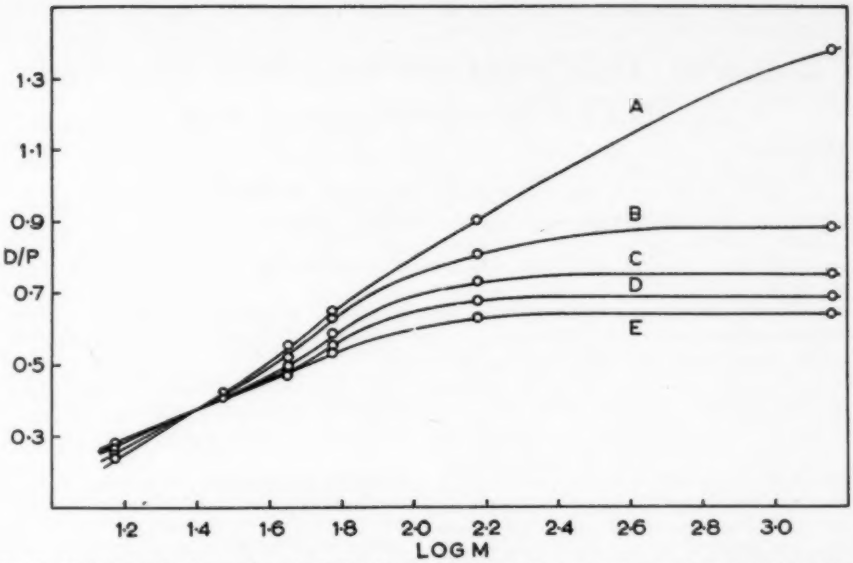

FIG. 8.—Variation of the dye-protein ratio (D/P) with the logarithm of the time in minutes (M) during which staining took place at the following protein concentrations. A: 5.22 g. per 100 ml., B: 3.48 g. per 100 ml., C: 2.09 g. per 100 ml., D: 1.04 g. per 100 ml., and E: 0.21 g. per 100 ml. Experimental conditions are described in the text.

being γ-globulin, whole serum and albumin (Figs. 1, 7). This agrees with the order observed by Gorringe (1957) at high lissamine green concentrations, and by Jencks *et al.* (1955) with bromophenol blue. Nevertheless, it is plain that conditions can usually be determined under which linearity of uptake can be accomplished over a large concentration range for a given fraction (Table II). Whether such conditions yield identical results for every fraction requires further experimentation; it involves the assumption that the staining properties of the applied commercial fractions and the separated physiological fractions are identical.

Comparison of Figs. 3 and 8 proves that greater staining efficiency is realized when dye and denaturant are separated. This is due to the adverse effect on the dye-protein ratio of increasing concentrations of the acid denaturant (Fig. 6). The expected correlation between uptake and dye concentration leads to the highest optimal dye-protein ratio of 0.46 obtained in these studies (Fig. 4, Table II); however, in terms of dye-protein ratio per dye concentration, it is the lowest and hence the least economical. It is perhaps surprising to note that at high protein concentrations, the uptake at 30° C. is about twice that at 10° C. (Fig. 5). This suggests that fluctuation in laboratory temperature can lead to disconcerting results and that thermostatic control of the staining procedure is advisable.

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A RAPID AND PRECISE METHOD FOR THE DETERMINATION OF UREA

BY

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A method is described for the determination of urea in plasma and urine. The effects of variations in the experimental conditions are examined and the results of recovery experiments and other tests of precision and accuracy are reported.

In comparison with other methods in current use, this method has distinct advantages in sensitivity, simplicity, and precision, thus economizing in time, sample volume, reagents, and equipment.

Although urea in biological fluids is frequently measured, a method has yet to be described which is precise, sensitive, and simple. Those most commonly used depend on the measurement of ammonium carbonate produced by hydrolysis with urease, or on the measurement of the yellow pigment formed by condensing diacetyl with urea. The best results are obtained by urease hydrolysis followed by aeration and titration of ammonia, but this requires large samples and is time consuming.

The reaction of ammonia with sodium phenate and hypochlorite to produce a blue colour (Berthelot, 1859; Thomas, 1912) was used by Borsook (1935) in the estimation of ammonium carbonate produced by hydrolysis of urea. Lubochinsky and Zalta (1954) found that the colour reaction was greatly improved in speed, yield, and reproducibility by the presence of sodium nitroprusside as a catalyst. Their discovery is adapted here for the determination of urea after hydrolysis with urease. The present method is as simple and rapid as techniques using Nessler's reagent or diacetyl, but has greater precision, similar to that of the aeration-titration procedure. It can be applied to as little as 10 μl. of plasma.

Method

Reagents.—Some distilled water may contain sufficient ammonia to make it unsuitable for the preparation of reagents and standards and for the dilution of samples. It is therefore necessary to redistil from all-glass apparatus or to use a cation exchange resin, e.g., Zeo-Karb 225, Na form. As an additional safeguard, the same batch of water is used for the preparation of the blanks and dilute standards and for the dilution of the samples.

Stock Urea Standard.—This is 1.071 g. of urea per litre, containing 500 mg. of urea nitrogen per litre, and may be checked by Kjeldahl analysis.

Dilute Urea Standards.—Dilutions of the stock standard of 1:250 and 1:100 containing 2 mg. and 5 mg. of urea nitrogen per litre.

Buffer.—Monopotassium dihydrogen phosphate, 6 g., and 2 g. of disodium monohydrogen phosphate per litre.

Buffered Urease Solution.—About 200 Sumner units (about 30 mg.) of special purity urease[1] per 100 ml. of a 1% solution of the buffer. This keeps for about a week in a refrigerator. Each day sufficient is removed for immediate requirements and any excess is discarded.

Sodium Phenate. — Phenol, A.R., 25.0 g., is dissolved in about 800 ml. of water, mixed with 78 ml. of 4.0 N-sodium hydroxide solution, and diluted to 1 litre. This keeps for at least a month in a refrigerator but should not be used when it turns brown. It must be brought to room temperature before use.

Sodium Nitroprusside, 1%.—This keeps for at least a month in darkness at room temperature.

Sodium Nitroprusside, 0.01%.—A 1:100 dilution of the 1% solution keeps for at least a week in darkness at room temperature.

Sodium Hypochlorite, about 0.02 N.—This is prepared by diluting a commercially available stock solution[2] and standardized iodometrically (Vogel.

*Leverhulme Research Fellow.

[1] Special purity urease is obtained from Sigma Chemical Co., U.S.A. (U.K. Agents: George T. Gurr Ltd., 136, New King's Road, London, S.W.6.)

[2] Sodium hypochlorite solution, 10-14% w/w available chlorine, is obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, and from British Drug Houses Ltd., Poole, Dorset.

1951) by adding excess acid and potassium iodide and titrating with sodium thiosulphate. A variation of ±20% in normality has no measurable effect on results. This solution keeps for at least a month in darkness at room temperature.

Urease Incubation.—The sample incubated with urease preferably contains between 1 and 5 μg. of urea nitrogen. Therefore plasma is diluted 1:100, or 1:500 if the concentration is known to be high, and urine is diluted 1:1,000 or 1:2,500. One millilitre portions of water, dilute standards, and dilute samples are measured into 6 in. by 1 in. test tubes. One millilitre of buffered urease solution is added to each, which is then placed in a 37° C. water-bath for five minutes or left at room temperature for 10 minutes.

An alternative procedure is to pipette 10 μl. portions of stock urea standard and of plasma directly into 2 ml. of urease solution and to incubate as above. This avoids prior dilution, but the precision is limited by that of the pipette. Repetitive measurements with a constriction pipette, calibrated to contain 10 μl., have a coefficient of variation of about 1%.

Preformed Ammonia.—In all methods depending on the quantitative hydrolysis of urea to ammonium carbonate, preformed ammonia contributes to the final measurement. It is customary to neglect plasma ammonia, but if accurate urea results are required on urine the ammonia nitrogen must be allowed for. Urinary ammonia is measured by the same procedure as that described, substituting water for the urease solution; 1 ml. of a 1:200 dilution is usually satisfactory.

Colour Development. — After incubation, the following solutions are added immediately after each other to each tube: 2 ml. of sodium phenate, 3 ml. of 0.01% sodium nitroprusside, and 3 ml. of sodium hypochlorite. Maximum colour development is greatest in the absence of direct sunlight and is complete in 15 minutes at 27° C. and 30 minutes at 19° C. Optical density at 630 mμ is then constant for at least 24 hours.

After subtraction of blank readings, optical density is directly proportional to urea concentration.

Results

The results presented here describe (*a*) the characteristics of the reaction, including the effect of variations in the experimental conditions; and (*b*) the precision and accuracy of the method, as measured by replicate analysis, recovery experiments, comparison with results by the aeration-titration technique, and tests for interference by other substances.

Characteristics of the Reaction.—The interaction of ammonia, phenate, and hypochlorite yields an absorption curve which is reported to have a maximum near 625 mμ. The exact position of this maximum depends upon such factors as the concentration of reagents and the presence of catalysts. With the present method, and a "unicam SP500" spectrophotometer, we found the maximum at 630 mμ. Ilford spectrum orange filters 607, in a Hilger Spekker absorptiometer, gave satisfactory results.

The relationship between concentration and optical density is linear. If the sample after incubation contains more than 6 μg. of ammonia nitrogen, the coloured solution may be diluted in water and measured against the similarly diluted blank. This is valid even in the presence of 30 μg. of ammonia nitrogen, which would be produced from a 1:100 dilution of plasma containing 300 mg. of urea nitrogen per 100 ml.

It is important that the colour reagents are added promptly after each other. For every minute interval between the addition of nitroprusside and hypochlorite the final optical density is diminished by about 1.5%.

The depression of optical density by direct sunlight may be of the order of 10%, but filament and strip lighting are without effect. Although precautions can be taken to subject standards and unknowns to equal sunlight, it is better to carry out the reaction where natural lighting is minimal or to develop the colour in darkness.

Temperature has important effects on the speed and intensity of colour development. Maximum optical density is attained in 30 minutes at 19° C., 20 minutes at 24° C., 15 minutes at 27° C., and five minutes in a 37° C. water-bath. In each case the density is then stable for at least 24 hours. Development in a 37° C. water-bath yields densities about 10% less than development at 20° C., standards and unknowns being influenced equally, provided that the interval between adding the reagents and transferring to the bath is constant. It is more convenient to develop colour at room temperature, except in emergency work when each tube is placed in the 37° C. bath immediately after adding the reagents and is left there for five minutes.

The phenate-hypochlorite reaction is influenced by *p*H, and it was necessary to incorporate buffers in applying it to Kjeldahl digests (Lubochinsky and Zalta, 1954) or to acid solutions for absorption of ammonia (Brown, Duda, Korkes, and Handler, 1957). In the present application the inclusion of a buffer has proved unnecessary.

It was not found possible to simplify the procedure by combining colour reagents, as the combinations are very unstable. The effect of decreasing the concentration of either sodium phenate or nitroprusside is to retard colour

development; the effect of an increase is to intensify the reagent blank. The ratio of sodium hydroxide to phenol is optimum for rapid colour formation. A variation of ±20% in the iodometric titre of fresh 0.02 N-hypochlorite solution has no effect on the speed and intensity of colour development. After about two weeks the solution may deteriorate, resulting in delayed colour development. This deterioration is not accompanied by a parallel change in iodometric titre.

Precision and Accuracy.—In assessing the precision and accuracy of this method, it has been compared with the urease-aeration-titration procedure, which we considered the most reliable. Standard urea solutions were included in each aeration series and the acid titres required by the unknowns were related to those required by the standards. For the purpose of comparison, urine ammonia concentrations were not subtracted in either method.

Table I compares the precision of the two methods. The errors of the present method were proportional to the urea concentration, while those of the aeration procedure were more constant irrespective of the quantity of ammonia produced. Table I thus shows that for normal or slightly raised plasma urea concentrations the precision was much better by the present method, whereas at higher concentrations the aeration procedure was slightly better.

TABLE I

PRECISION OF UREASE-PHENATE-HYPOCHLORITE AND UREASE-AERATION-TITRATION METHODS

	Present Method (%)	Aeration Method (%)
Standard urea solutions ..	1·0*	0·8
Plasma samples containing less than 50 mg. urea N/100 ml.	1·2	4·0
Plasma samples containing more than 50 mg. urea N/100 ml.	1·0	0·8
Urine samples	1·0	0·8

* Values reported are coefficients of variation $\left(\frac{\text{S.D.}}{\text{Mean}} \times 100\right)$ calculated in each case from duplicate results on 50 samples.

Table II compares the results obtained by the two techniques on 18 plasma and 15 urine samples. Agreement was generally good, the most significant discrepancies occurring in plasma samples of low urea concentration, where the aeration procedure was less reliable.

Table III reports the results of recovery experiments on five specimens of plasma and five of urine. Most recoveries were correct within 1%. Mean recoveries were 100% by the present method and 99.3% by aeration.

The recovery experiments and the good correlation with the aeration method suggested that there was unlikely to be significant interference by substances commonly encountered in plasma or urine. Nevertheless, five substances that might interfere were investigated, namely, glucosamine, glutamine, and citrulline, which readily liberate ammonia, and bilirubin and haemoglobin. Each of these substances was added to diluted plasma in concentrations shown in Table IV. The three labile amines caused no interference, and the interference by high concentrations of bilirubin was small. Interference by

TABLE II

COMPARISON OF RESULTS BY THE UREASE-PHENATE-HYPOCHLORITE AND UREASE-AERATION-TITRATION METHODS ON 18 SAMPLES OF PLASMA AND 15 SAMPLES OF URINE

Present Method (A)	Aeration Method (B)	Difference (A−B)	Percentage Difference $\left(\frac{A-B}{A} \times 100\right)$
Plasma containing less than 50 mg. urea N per 100 ml.			
11·8*	11·1	+0·7	+5·9
11·4	13·1	−1·7	−14·9
12·6	12·4	+0·2	+1·6
12·9	13·3	−0·4	−3·1
20·5	20·8	−0·3	−1·5
44·4	44·7	−0·3	−0·7
46·7	47·3	−0·6	−1·3
Mean difference ..		−0·3	−2·0
Mean deviation† ..		0·6	−4·1
Plasma containing more than 50 mg. urea N per 100 ml.			
51·5	51·5	0	0
55·3	56·3	−1·0	−1·8
62·2	62·0	+0·2	+0·3
68·1	68·5	−0·4	−0·6
78·6	79·8	−1·2	−1·5
83·0	82·8	+0·2	+0·2
94·5	96·3	−1·8	−1·9
98·5	97·9	+0·6	+0·6
138·0	140·0	−2·0	−1·4
148·0	151·0	−3·0	−2·0
151·4	153·1	−1·7	−1·1
Mean difference ..		−0·9	−0·8
Mean deviation† ..		1·1	1·0
Urine			
206	210	−4	−1·9
215	213	+2	+0·9
305	307	−2	−0·7
378	384	−6	−1·6
405	409	−4	−1·0
455	459	−4	−0·9
469	466	+3	+0·6
587	591	−4	−0·7
590	600	−10	−1·7
638	646	−8	−1·3
648	657	−9	−1·4
762	740	+22	+2·9
758	761	−3	−0·4
810	822	−12	−1·5
840	812	+28	+3·3
Mean difference ..		−1	−0·4
Mean deviation† ..		8	1·4

* Values reported are means of duplicate results in mg. of urea N per 100 ml.
† Disregarding sign.

TABLE III

RECOVERY OF UREA* BY UREASE-PHENATE-HYPOCHLORITE AND UREASE-AERATION-TITRATION METHODS

Initial Concentration	Recovery by Present Method	Recovery by Aeration Method
Plasma		
11	251	251
13	247	248
21	251	251
51	254	248
138	249	243
Mean recovery for plasma	250	248
Mean deviation† from 250 mg./100 ml.	2	3
Urine		
206	249	249
215	252	245
378	253	251
387	251	250
405	243	248
Mean recovery for urine . .	250	249
Mean deviation† from 250 mg./100 ml.	3	2

* Urea recovered from five samples of plasma and five samples of urine when urea was added to increase the concentration by 250 mg. N per 100 ml. Values reported are means of duplicate results in mg. of urea N per 100 ml.

† Disregarding sign.

TABLE IV

TESTS FOR INTERFERENCE BY GLUCOSAMINE, GLUTAMINE, CITRULLINE, BILIRUBIN, AND HAEMOGLOBIN

Added Substance	Concentration in Plasma (mg./100 ml.)	Plasma Urea N (mg./100 ml.)	Plasma Dilution	Apparent Increase in Urea N (mg./100 ml.)
Glucosamine . .	20	17	1:100	0
Glutamine	20	92	1:100	0
Citrulline	20	92	1:100	0
Bilirubin	10	22	1:100	+0·3
	25	151	1:500	+1·5
	40	88	1:500	+0·7
	50	34	1:100	+0·1
Haemoglobin	75	65	1:500	−0·4
	75	92	1:500	−0·4
	150	13	1:100	+2·0
	150	17	1:100	+0·5
	150	65	1:500	+0·7
	150	92	1:500	+0·3
	200	65	1:500	+2·1
	200	92	1:500	+1·2
	250	128	1:500	−0·8
	300	13	1:100	+2·7
	300	92	1:500	+1·2
	300	119	1:500	−2·1
	600	119	1:500	−3.0

haemoglobin was greater and unpredictable, being positive at low urea levels and negative at high ones. This indicates that accuracy is reduced in the presence of haemolysis.

All methods employing urease followed by measurement of the products of hydrolysis yield a result with plasma even in the absence of the enzyme. This may be due partly to plasma ammonia, and possibly other substances, and partly to non-enzymic hydrolysis of urea. In a series of 10 plasma samples stored in a refrigerator up to 30 hours and containing from 8 to 140 mg. of urea nitrogen per 100 ml., this was found to correspond to an average of 0.4 mg. of nitrogen per 100 ml. by both the new and aeration methods. Three plasma samples which had been stored in a refrigerator for two months gave results of 1.6 to 2.9 mg. of nitrogen per 100 ml.

Discussion

The great sensitivity of the colour reaction permits the measurement of very small amounts of urea in small samples, which therefore require very little urease. Special purity urease is to be preferred to cruder preparations because of the lower blanks, maximum specificity, and absence of insoluble material which would require centrifuging. Although the special purity enzyme is expensive, so little is used that the cost of reagents with this method is about one-fifth of that using commercial urease tablets and aeration.

The precision of this method is as good as that of the best routine procedure. There is, however, one hazard arising from the sensitivity of the colour reaction, and this is the unintentional use of distilled water containing ammonia, for example, from a wash bottle.

The present method is about as quick as the Nesslerization and diacetyl procedures and is considerably quicker than the aeration technique. This remains true if one takes into account time spent in preparing reagents and cleaning apparatus. In the presence of exceptionally large quantities of urea, it has a great advantage over other methods because one can validly dilute the final solution for absorptiometry instead of having to repeat the assay from the beginning. It is also convenient that, without any special modification, this procedure is equally suitable for plasma, urine, and other biological material.

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A SIMPLE AND RAPID METHOD FOR THE SIMULTANEOUS DETERMINATION OF CALCIUM AND MAGNESIUM FROM THE SAME SAMPLE OF BLOOD SERUM

BY

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A simple and rapid procedure has been developed for the complexometric titration of serum calcium and magnesium using "plasmocorinth B" as indicator. Both determinations can be carried out from the same 0.5 ml. sample. The method is in good agreement with the established calcium and magnesium methods.

The generally used classic methods for the determination of serum calcium (Kramer and Tisdall, 1921) and magnesium (Denis, 1922) take time, and the results are obtained after a delay of one or two days, so hindering progress in the knowledge of the role played by these ions in physiology and pathology. Simpler methods were therefore sought for their determination. New methods were developed (Kingsley and Schaffert, 1953; Tsao, 1952; Natelson and Penniall, 1955; Greenberg and Mackey, 1932; Davis, 1955; Hirschfelder and Serles, 1934; Orange and Rhein, 1951; Vallee, 1954), but most of them were either cumbersome or inaccurate or required special equipment. Complexometric titration (Schwarzenbach, Biedermann, and Bangerter, 1946) with disodium ethylenediaminetetra-acetate (E.D.T.A.) seemed to be the most suitable among these procedures, although most of the indicators in use did not prove adequate, as the end-point of the titration was not sufficiently sharp and could not be established by simple visual observation. Recently new and suitable indicators have been reported for the simple determination of calcium (Diehl and Ellingboe, 1956; Patton and Reeder, 1956; Hildebrand and Reilley, 1957).

Yanagisawa (1955) and Kingsley and Robnett (1957) described a simple colorimetric procedure for the determination of serum calcium using the dye "plasmocorinth B" (Inabata Industry and Co. Ltd., Osaka, Japan). This dye is chemically very similar to the compounds recently recommended as indicators for the complexometric titration of calcium. The latter are derivatives of naphthyl-azo-naphthalene, whereas "plasmocorinth B" is a naphthyl-azo-benzene (disodium-1-hydroxy-4-chloro-benzene-(2-azo-2)-1,8 dihydroxy-naphthalene-3,6-disulphonic acid).

In the course of preliminary experiments it was found that "plasmocorinth B"* may also be used as an indicator for the complexometric titration of calcium and magnesium. If E.D.T.A. is added in the presence of sodium hydroxide to a solution containing calcium and magnesium ions the dye, after having bound equivalent amounts of calcium, forms a bluish-violet soluble complex with E.D.T.A.; magnesium does not interfere with this reaction. In the presence of ammonium hydroxide, on the other hand, E.D.T.A. reacts with "plasmocorinth B" only after having bound both calcium and magnesium (Kovács and Tárnoky, 1959a).

This paper deals with a method for the determination of serum calcium and magnesium based on this principle, and a simple titrimetric determination for urinary calcium and magnesium developed from this method will be reported later.

Materials and Methods

Reagents

0.001 M E.D.T.A. Solution.—Dissolve 0.3722 g. E.D.T.A. in 1,000 ml. distilled water. Standardize against $MgSO_4.7H_2O$.

0.5 N NaOH.—Turbid solutions containing carbonate should not be used.

*Supplied by the courtesy of Inabata Industry and Co. Ltd.

Buffer Solution.—Dissolve 0.83 g. NH_4Cl in a small amount of water, add 12 ml. of concentrated NH_4OH, and fill to 100 ml. with distilled water.

0.5 N HCl.

Indicator Solution.—Dissolve 10 mg. of "plasmocorinth B" in 100 ml. distilled water. Keep the solution in a dark bottle; it can be used for at least six months.

The water used at the preparation of the solutions must be free of metal ions (twice-distilled water). The solutions should be preferably kept in polyethylene bottles. All glassware should be rinsed with twice-distilled water before use.

Procedure.—To 0.5 ml. of blood serum in a 50-ml. beaker add in succession 5 ml. distilled water, 1.5 ml. 0.5 N NaOH, and 1 ml. of the indicator solution. Titrate with 0.001 M E.D.T.A. from a 2-ml. microburette to the first appearance of a bluish-violet colour. This end-colour develops sharply and does not change if more E.D.T.A. is added to the solution. Note the burette reading.

Add 1.2 ml. 0.5 N HCl to the same sample and mix thoroughly; the colour of the indicator will turn again to red. Pipette 1.5 ml. buffer solution to the sample and continue the titration to the first appearance of a light blue colour which also develops sharply and does not change on the addition of excess E.D.T.A.

Calcium is determined by the first titration and magnesium by the second. In the case of serial determinations care should be taken that not more than five to 10 minutes should elapse between the addition of NaOH and the beginning of the titration.

Calculation.—One millilitre of 0.001 M E.D.T.A. solution is equivalent to 0.04008 mg.=2 μEq. calcium and 0.02432 mg.=2 μEq. magnesium respectively. Thus, the concentration of serum calcium and magnesium can be obtained according to the formula:

ml. (E.D.T.A. solution consumed for Ca)×8=Ca (mg./100 ml.)

ml. (consumed for Mg)×4.86=Mg (mg./100 ml.)

or

ml. (consumed for Ca)×4=Ca (mEq./l.)

ml. (consumed for Mg)×4=Mg (mEq./l.)

Results

The accuracy of the method was tested by comparing it with the oxalate method for calcium (Kramer and Tisdall, 1921) and the phosphate method for magnesium (Denis, 1922) determination (Table I).

With each method duplicate or triplicate determinations were made on every serum sample and the means of these values are given in Table I. (Many of the sera analysed were taken from uraemic patients, and some of them were haemolysed; presumably this may be the cause of

TABLE I

COMPARISON OF PROPOSED AND OXALATE (KRAMER AND TISDALL, 1921) AND PHOSPHATE METHODS (DENIS, 1922)

Serum Sample	Ca (mg./100 ml.)		Difference	Mg (mg./100 ml.)		Difference
	Oxalate Method	Proposed Method		Phosphate Method	Proposed Method	
1	10·4	10·4	0·0	2·48	2·50	+0·02
2	10·9	10·9	0·0	4·10	4·15	+0·05
3	10·3	10·3	0·0	2·80	2·90	+0·10
4	10·3	10·2	−0·1	2·75	2·90	+0·15
5	9·3	9·4	+0·1			
6	9·0	9·1	+0·1			
7	9·9	9·7	−0·2			
8	10·1	9·8	−0·3			
9	9·9	9·7	−0·2	3·60	3·78	+0·18
10	10·0	9·7	−0·3	2·76	3·00	+0·24
11	10·5	10·4	−0·1	3·82	4·00	+0·18
12	9·7	9·5	−0·2	4·00	4·20	+0·20
13	11·4	11·0	−0·4	2·52	2·75	+0·23
14	9·4	9·3	−0·1	2·90	2·75	−0·15
15	8·8	8·6	−0·2	3·32	3·30	−0·02
16	9·1	8·8	−0·3	3·02	2·98	−0·04
17	9·8	9·6	−0·2	3·10	2·92	−0·18
18	8·9	8·9	0·0			
19	9·8	9·9	+0·2	3·20	3·02	−0·18
20	10·7	10·8	+0·1	2·62	3·00	+0·38
21	8·4	8·3	−0·1	2·54	2·70	+0·16
22	9·3	9·2	−0·1	2·46	2·50	+0·04
23	9·6	9·4	−0·2	2·60	2·55	−0·05
24	9·5	9·5	0·0	1·95	2·15	+0·20
25	8·4	8·2	−0·2	2·45	2·60	+0·15
26	10·1	10·0	−0·1	1·95	2·30	+0·35
27	10·1	9·9	−0·2			
28	9·3	9·4	+0·1	2·80	2·78	−0·02
	Average of the difference ..		−0·1	Average of the difference ..		+0·09

TABLE II

RECOVERY OF ADDED CALCIUM AND MAGNESIUM FROM POOLED SERUM

Sample No. :	1	2	3	4	5	6	7	8	9
Ca in sample (μg.) ..	52·4	52·4	52·4	52·4	52·4	52·4	52·4	52·4	52·4
Ca added (μg.) ..	0·0	52·9	0·0	52·9	102·5	0·0	102·5	102·5	52·9
Ca found (μg.) ..	52·4	103·0	53·4	106·1	155·0	54·6	155·3	155·1	105·9
Ca calculated (μg.) ..	52·4	105·3	52·4	105·3	154·9	52·4	154·9	154·9	105·3
$\frac{\text{Ca found}}{\text{Ca calculated}}$ (%) ..	100·0	97·5	102·0	100·6	100·1	104·2	100·4	100·1	100·4
Mg in sample (μg.) ..	14·5	14·5	14·5	14·5	14·5	14·5	14·5	14·5	14·5
Mg added (μg.) ..	0·0	0·0	17·4	17·4	0·0	33·5	33·5	17·4	33·5
Mg found (μg.) ..	14·5	15·1	30·7	31·8	14·9	48·9	49·2	32·5	48·1
Mg calculated (μg.) ..	14·5	14·5	31·9	31·9	14·5	48·0	48·0	31·9	48·0
$\frac{\text{Mg found}}{\text{Mg calculated}}$ (%) ..	100·0	104·2	96·3	99·7	102·9	102·0	102·8	102·0	100·2

the high magnesium concentration found in some of the samples.) Good agreement was obtained with the different methods.

Varying amounts of calcium and magnesium were added to 0.5 ml. samples of pooled serum and the specimens were titrated as described above. The recovery of the added calcium ranged from 97.5 to 104.2%, with an average of 100.4%, and for magnesium the recoveries ranged from 96.3 to 104.2%, with an average of 101.3% (Table II).

The precision of the method was determined by calculating the standard deviations (Copeland, 1957) of 25 duplicate determinations. Thus, values for calcium determination, S.D. = ±0.23 mg./100 ml., and for magnesium, S.D. = ±0.20 mg./100 ml., were obtained.

Addendum

Since the completion of this work a complexometric method for serum calcium has been published using " eriochromblue SE " (Geigy AG., Basel, Switzerland) as indicator (Flaschka, Abd El Raheem, and Sadek, 1958). According to our information this substance is chemically identical with " plasmocorinth B." Several determinations have proved that " eriochromblue SE "* is just as suitable as " plasmocorinth B " in the above procedure.

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A SIMPLE METHOD FOR THE DETERMINATION OF SERUM HAPTOGLOBINS

BY

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Changes in the serum haptoglobin level have been reported in certain forms of anaemia (Allison and ap Rees, 1957; Allison, 1958; Nosslin and Nyman, 1958; Nyman, Gydell, and Nosslin, 1959), in hepatobiliary disease (Owen, Mackay, and Got, 1959) and in various inflammatory and chronic diseases (Jayle and Boussier, 1955; Allison and Blumberg, 1958). A simple colorimetric procedure for the determination of serum haptoglobins is presented. It is based on the peroxidase activity of haptoglobin-methaemoglobin complexes.

Reagents and Apparatus

Guiacol Reagent (Connell and Smithies, 1959).—Guiacol (analytical grade), 3.72 g., is dissolved in 700 ml. water to which is added 100 ml. of 1 M-acetic acid. The *p*H of the mixture is then adjusted to 4.0 by the addition of 1 M-sodium hydroxide, using a glass electrode *p*H meter. Finally the volume is made up to 1,000 ml.

Hydrogen Peroxide, 0.05 M.—This is prepared immediately before use by diluting a stock solution standardized by titration against standard potassium permanganate.

Methaemoglobin Solution.—To 8 vol. human erythrocytes, previously washed three times with saline, is added 3 vol. of water and 1 vol. of ether. The mixture is shaken and centrifuged, and the clear haemolysate pipetted off. The concentration of haemoglobin in the haemolysate is determined, using one of the standard methods for the determination of haemoglobin in blood and the solution diluted until the haemoglobin concentration is 1 g./100 ml. To 25 ml. of this solution is added 10 ml. of potassium ferricyanide (100 mg./100 ml.) to convert the haemoglobin to methaemoglobin. After 10 min. the volume is made up to 500 ml. The solution can be stored for several weeks at 0° C.

Sodium Chloride, 0.15 M.

Procedure.—The test serum (1 vol.) is diluted with saline (4 vol.). To 1 ml. aliquots of diluted serum is added 1 ml. of methaemoglobin solution and 1 ml. of water respectively. Two test-tubes, each containing 5 ml. of guaicol reagent, are placed in a water-bath fitted with a lid and set at 25° C. After 10 min., 0.1 ml. of the mixture of diluted serum and methaemoglobin solution is added to the first tube and 0.1 ml. of the mixture of diluted serum and water, as a blank, to the second. Immediately thereafter 1.0 ml. of hydrogen peroxide, previously warmed to 25° C., is added to each tube and the contents of the tubes quickly mixed. After eight minutes the tubes are removed from the bath and the optical densities of the solutions measured in a photoelectric colorimeter at a wavelength of 470 mμ or using a blue filter. The instrument is set to zero with water and readings are made within four minutes. With multiple tests, timing is such that all tubes are treated similarly. The coloured material formed during the reaction (tetraguiacol) fades slowly if exposed to bright daylight. The lid of the water-bath should therefore be closed during the development of the colour and tubes should be kept in the dark until readings are made.

The concentration of haptoglobin is obtained from a calibration curve and is expressed in terms of bound methaemoglobin. If readings are too low or too near the maximum (Fig. 1), the test should be repeated using less diluted or more diluted serum. In this case, values from the calibration curve must be divided or multiplied accordingly.

The calibration curve is constructed as follows:

Portions (1.0 ml.) of methaemoglobin solution are placed in each of 11 tubes. To these are added respectively increasing amounts of (0.0, 0.1, 0.2, 0.3, . . . 1.0 ml.) pooled normal serum free from haemolysis. Saline is then added, where necessary, so that the total volume in each tube is 2.0 ml. Each sample and a blank prepared with 0.1 ml. NaCl and 5 ml. guiacol is then submitted to the procedure described above. The photometer is set to zero with the blank. The readings are plotted against the amount of serum in each reaction tube (Fig. 1). The point of inflexion in the curve obtained indicates the amount of undiluted pooled serum which just binds all the methaemoglobin present since the addition of more serum does not significantly influence the readings (see Connell and Smithies, 1959). Thus, the reading at

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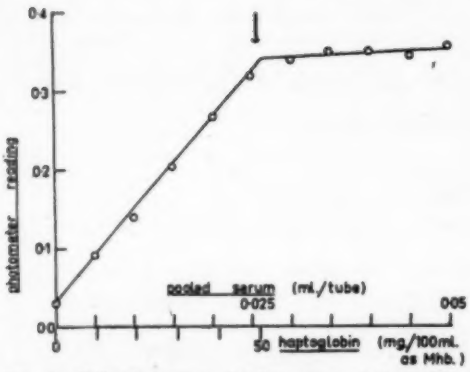

FIG. 1.—Calibration curve. Readings were obtained with a "unicam" SP1400 spectrophotometer at a wavelength of 470 mμ using 10 mm. diameter cuvettes. The arrow indicates the point of inflexion. The haptoglobin concentrations (lower scale on abscissa) are those of undiluted serum. With test sera, which are diluted, the values should be multiplied by the dilution factor. Normally this is 5 (see text).

this point corresponds to a methaemoglobin-binding capacity of 50 mg./100 ml. Test sera, however, are normally diluted 1 in 5 so that, with these, the reading corresponds to a haptoglobin content of 250 mg./100 ml. (as methaemoglobin). The sloping part of the curve relates lower readings to the corresponding haptoglobin contents (Fig. 1).

Discussion

Polonovski and Jayle (1940) demonstrated that under certain conditions the peroxidase activity of the haemoglobin-haptoglobin complex is much greater than that of free haemoglobin. A similar phenomenon occurs with methaemoglobin (Connell and Smithies, 1959) and this forms the basis of the present method. By subtracting the reading for the serum blank from the test reading allowance is made for the reagent blank, for non-enzymatic oxidation of guiacol, and for the peroxidase activity of the serum itself, which in some cases is appreciable. This is presumably due to the presence of verdo-peroxidase from leucocytes (Agner, 1941). Methaemalbumin, if present, will also contribute to the serum blank. Under the conditions chosen, free methaemoglobin has a peroxidase activity about 10% of that of the methaemoglobin-haptoglobin complex (Fig. 1). However, allowance is made for the activity of any uncombined methaemoglobin in the construction of the calibration curve.

The sensitivity of the guiacol reagent slowly increases on standing, and a calibration curve should be made each week.

Sera should be free from haemolysis and should not be diluted if the haptoglobin content is below 40 mg./100 ml.

Calibration of the method in terms of methaemoglobin means that results obtained by different laboratories should be comparable since it is generally possible to measure haemoglobin, from which the methaemoglobin is obtained, with reasonable precision. Eventually it should be possible to standardize calibration curves using pure haptoglobin solution. The quantities employed in constructing the calibration curve have been calculated on the basis of a mean normal serum haptoglobin concentration of around 100 mg./100 ml. (Jayle and Boussier, 1955; Laurell and Nyman, 1957; Nyman, 1958; Owen *et al.*, 1959). Thus, approximately 0.025 ml. of pooled normal serum should be equivalent to 0.05 ml. of the methaemoglobin solution (50 mg./100 ml.). We have found that the intrinsic peroxidase activity of pooled normal serum is sufficiently low to be neglected, especially if the sample has been stored frozen solid for some days.

The method described here for the estimation of serum haptoglobins gives more precise values than the simple electrophoretic methods used by Laurell and Nyman (1957) and by Owen *et al.* (1959) and is quicker than the electrophoretic method described by Lathem and Worley (1959). The procedure described by Jayle (1951) gives precise values but involves a titration carried out with very careful timing and utilizes a reagent (ethyl hydroperoxide) which is potentially explosive (Baeyer and Villiger, 1901; Tobolsky and Mesrobian, 1954). The method devised by Connell and Smithies (1959) is likewise precise, but involves, for each sample, the taking of several readings at short intervals and requires a spectrophotometer fitted with a thermostatically controlled chamber.

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INFECTION WITH DIFFERENT SPECIES OF LEPTOSPIRES IN HUSBAND AND WIFE

BY

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Leptospirosis occurring in a husband and wife is described. Both were responsible for cleaning up the excreta of a recently acquired puppy. The wife developed leptospirosis canicola, and three months later the husband developed leptospirosis icterohaemorrhagica. Difficulty was experienced in ascertaining the infecting species of leptospire in the husband owing to the agglutination tests giving an equal rise and fall in titres for both *L. icterohaemorrhagiae* and *L. canicola*. The diagnosis was only confirmed after cross-absorption tests had been done.

The husband developed pulmonary complications on the nineteenth day. The radiological appearances of the chest are discussed, together with a brief review of the literature on the radiological manifestations of pulmonary lesions in Weil's disease.

Although more than 40 different strains of leptospires have been recognized by serological methods, only *Leptospira icterohaemorrhagiae*, which produces Weil's disease, and *Leptospira canicola*, the common spirochaetal infection of dogs, are normally associated with human infection in Britain. Keal (1957) has, however, reported a case of leptospirosis due to *Leptospira grippotyphosa* occurring in a Malayan who had arrived in England by air from Malaya 10 days previously. Leptospirosis icterohaemorrhagica is relatively common, especially among farm workers, sewer workers, miners, and fish cleaners. Since the description by Baber and Stuart (1946) of the first recognized human infection with *L. canicola* in this country, 30 cases occurring in Britain have been reported in the literature. Broom (1951a) noted that although 15 to 20 cases of *L. canicola* were recognized annually in Britain, the incidence was certainly higher.

The serological diagnosis of leptospirosis is generally straightforward, but occasionally it may be equivocal or definitely misleading. Case 1 illustrates the difficulty that may be experienced in determining the exact species of leptospire responsible for the disease, especially when the clinical picture and logical sequence of events also point to a species other than the true culprit. Of further interest are the pulmonary complications in Case 1.

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Case Reports

Case 1.—A miner, aged 50, was admitted to hospital on May 2, 1955. He had been in good health until six days previously when he complained of marked weakness and of pains in the lumbar region and calves of the legs. The following day he had severe frontal and occipital headache, and rigors which occurred several times each day until admission. Before admission to hospital he had been working on the surface at a colliery for a year, and he was quite emphatic that there were no rats near his place of work.

In December, 1954, the patient bought a one-month-old puppy; for four months both he and his wife frequently mopped up pools of urine left by the dog.

On admission the temperature was 101° F. with a pulse rate of 104 per minute. He appeared ill and the conjunctivae were obviously injected. Moderate neck stiffness and a positive Kernig's sign were present. A few small abrasions were noted on the fingers of both hands and the axillary lymph glands were enlarged. Two days later neck and back stiffness had become pronounced.

Investigations.—A blood count showed 7,200 leucocytes per c.mm. (polymorphs 56%, lymphocytes 30%, monocytes 14%). Haemoglobin was 13 g.%, the E.S.R. 46 mm. in the first hour. The cerebrospinal fluid was slightly opalescent, with 138 cells per c.mm. (lymphocytes 90%), total protein 165 mg. per 100 ml. with a moderate excess of globulin, chlorides 700 mg. per 100 ml., sugar 44 mg. per 100 ml.; no organisms were seen on direct examination and on culture it was sterile; the Wassermann reaction was negative.

The urine showed a cloud of albumin, but centrifuged deposit showed only one granular cast per 20 high-power fields. Blood urea was 30 mg. per 100 ml., serum bilirubin 0.4 mg. per 100 ml., serum alkaline phosphatase 12 units, thymol turbidity 2 units per 100 ml., and a colloidal gold test was negative. Blood culture was sterile. A radiograph of the chest two days after admission showed a slight diffuse mottling due to pneumoconiosis.

The serum agglutination reactions were as follows:

Date of Specimen	*L. canicola*	*L. icterohaemorrhagiae*
3.5.55	1/300	1/300[1]
11.5.55	1/1,000	1/300[2]
		Trace at 1/1,000
29.5.55	1/1,000	1/1,000[2]
	(Partial) 1/3,000	(Partial) 1/3,000
18.7.55	1/1,000	1/1,000[2]
5.11.55	1/300	1/300[2]
1.4.56	1/300	1/300[2]
15.7.57	1/30	1/100[2]

[1] Tested by Dr. R. W. Harvey. [2] Tested by Dr. J. C. Broom.

On May 29, 1955, a cross-absorption test was performed by Dr. J. C. Broom, who reported that the results indicated that the patient was suffering from true Weil's disease, but that the results were not as clear cut as he really liked, and he therefore suggested a further cross-absorption test in one month. A further specimen of blood taken on July 18, 1955, was reported on by Dr. Broom as follows:

> "After absorption with *L. icterohaemorrhagiae* the serum was negative with both *L. icterohaemorrhagiae* and *L. canicola* from 1/30 upwards (this was the lowest dilution tested). After absorption with *L. canicola*, it was negative with *L. canicola* from 1/30 upwards, but still agglutinated *L. icterohaemorrhagiae* to 1/300 with a trace at 1/1,000."

Unfortunately the puppy could not be investigated as the patient had ordered his wife to have it destroyed after overhearing a discussion in the ward about the animal.

Treatment with penicillin, 500,000 units eight-hourly, was begun on the second day after admission and continued for 10 days. His condition improved, but on the nineteenth day of admission he complained of a severe, sharp stabbing pain on both sides of the chest, which was aggravated by deep breathing. Examination of the chest revealed signs of an area of consolidation at both bases. Treatment with oxytetracycline, 500 mg. six-hourly, was begun. On the following day the temperature rose to 100° F. (37.8° C.) and it remained raised for the ensuing four days. The patient then gradually improved, but the impression was formed that the antibiotic treatment was playing little or no part in the improvement.

Further investigations at this stage: Blood count, 4,000 leucocytes per c.mm. (polymorphs 64%, lymphocytes 27%, monocytes 9%); E.S.R. 20 mm. in the first hour. No pathogenic organisms were found in several specimens of sputum and no cold agglutinins were detected in serum. A chest radiograph taken in the ward two days after the onset of chest pain showed small areas of patchy consolidation over both lower zones with a small pleural effusion at the left base. Nine days later, a further chest radiograph showed that the effusion had cleared, but that there were still small irregular areas of consolidation in the left lower lobe and lingula. A radiograph on June 16 still showed a small area of consolidation in the lingula. A final chest radiograph taken six months later was normal, apart from slight mottling due to pneumoconiosis.

Case 2.—A housewife, aged 48, the wife of Case 1, developed severe frontal headache and pyrexia in February, 1955. The following day she complained of general weakness and of pains in the calves of the legs and pain and stiffness in the back. She had marked photophobia and vomited frequently. (She ascribed her condition to "gastric flu.") She could not remember if she had had any cuts or scratches on her hands at the time of the illness. After two weeks her condition slowly improved, but she did not feel really well for a further three weeks.

Examination three weeks after her husband's admission to hospital revealed no abnormal physical signs.

A serum agglutination reaction on June 25, 1955, was reported on by Dr. Broom as:

> "The patient's serum is positive to 1/300 with a trace at 1/1,000 with *L. canicola* and shows only traces of agglutination in low dilutions with *L. icterohaemorrhagiae*."

Serum from the son, aged 24, and daughter, aged 17, of Cases 1 and 2 failed to agglutinate suspensions of *L. icterohaemorrhagiae* and *L. canicola* (Dr. R. W. Harvey). Neither the son nor the daughter had ever cleaned up the excreta from the puppy.

Discussion

In leptospirosis agglutinins normally appear about the end of the first week after the onset of symptoms, and reach a maximum between the third and fifth week of the disease, but Broom (1951b) mentions a few cases in which antibody production was delayed for more than three weeks. In the early stages of leptospirosis a patient's serum not uncommonly agglutinates more than one species of leptospire. Occasionally the titre of agglutination for a related heterologous type may be as high or even higher than the titre for the infecting species (Gispen and Schüffner, 1939). As the disease progresses the titres for the infecting species usually rise, while the heterologous titres fall. In Case 1 the heterologous titre remained as high as the homologous titre for at least 11 months, which is exceptional (Broom, personal communication); indeed, the correct diagnosis was only obtained after cross-absorption tests had been done. A diagnosis

based on an agglutination test only may be misleading as in the case recorded by O'Connell and Broom (1952).

The clinical evidence in Case 1 pointed to a leptospirosis canicola infection. No history of contact with rats could be obtained. Though classed as a miner, he had been working on the surface at the colliery as a rider, i.e., he was responsible for transporting slack in special wagons, and he was quite adamant that his place of work was dry and free from rats. However, the waste slack from underground could have been contaminated with rats' urine. It appears certain that his wife (Case 2) had had leptospirosis canicola three months before his own illness; yet both husband and wife shared the duty of mopping up pools of urine left by the puppy. The animal was apparently quite fit. Broom and MacIntyre (1948) have estimated that approximately 25 to 40% of dogs in England become infected with *L. canicola* at some period during their lifetime. Many of these dogs are symptomless carriers. It was not possible to prove that the puppy in this case was a carrier of *L. canicola*, as it was unfortunately destroyed without our knowledge.

The pulmonary manifestations of leptospirosis do not appear to have been generally recognized in Britain. (A more detailed description of the radiological appearances of pulmonary lesions in leptospirosis will be described elsewhere.) Moeschlin (1943), in Switzerland, described four cases of leptospirosis icterohaemorrhagica which, on radiological examination, showed bilateral patchy pulmonary infiltration. Extensive haemorrhage into the lung parenchyma was found in one patient at necropsy. Radiological signs of areas of patchy consolidation in both lungs were also described by Havens, Bucher, and Reimann (1941) in two out of seven men with confirmed Weil's disease. One of the two cases died and at necropsy both lungs showed a patchy haemorrhagic type of consolidation in the lower lobes. "Fleeting lung infiltrations" in the absence of physical signs were noted by Gsell (1949). Silverstein (1953), in a review of the radiological findings in Weil's disease, found radiological evidence of "pulmonary infiltrations" in four out of 33 patients with confirmed leptospirosis icterohaemorrhagica. Three cases were described in detail with reproductions of the chest radiographs. The presence of small irregular mottling over the lower and middle zones of both lungs was evident in the chest radiograph of two cases. A third case had a large area of apparent consolidation at the right lower lobe; at necropsy, however, both lungs were generally oedematous, and on microscopy the alveoli were seen to be filled with red blood cells, the right lower lobe appearing no different from the rest of the lung. It is interesting to note that several of the reported cases of Weil's disease first presented themselves as cases of acute respiratory infection.

The literature on the pathology of pulmonary lesions in leptospirosis is scanty, but a study of the necropsy findings reveals a fairly consistent picture. In severe cases the lungs are oedematous and the alveoli packed with red blood cells. The haemorrhagic areas may be small and localized or they may be extensive; they are generally bilateral, though rarely symmetrical. Occasionally small effusions are present. The cause of these haemorrhagic areas in the lung is uncertain. Haemorrhagic manifestations such as petechiae, epistaxis, haemoptyses, haematemesis, and melaena are not uncommon in Weil's disease. At necropsy large haemorrhages may be found in the intestinal wall, while in the kidneys there may be subcapsular and capillary haemorrhages. Experimental infection of guinea-pigs with *L. icterohaemorrhagiae* results in widespread haemorrhages in the lung, due to capillary damage.

The possibility that the pulmonary lesions in the first patient were due to an intercurrent bronchopneumonia cannot be entirely ruled out. The radiological picture was not typically that of bronchopneumonia, but was very similar to some of the reported cases of leptospirosis with pulmonary manifestations. The white cell count was not raised nor was the erythrocyte sedimentation rate at the time of the lung complication, and no pathogenic organisms were found in several specimens of sputa.

If patients employed in occupations commonly associated with the presence of rats present with acute chest infections, and where the chest radiological appearances are similar to those described above, the diagnosis of Weil's disease is always worth considering.

I wish to thank Dr. J. C. Broom, of the Wellcome Laboratories of Tropical Medicine, and Dr. R. W. S. Harvey, of the Public Health Laboratories, Cardiff, for permission to quote their agglutination results.

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THE COLICINE TYPING OF COLIFORM BACILLI IN THE STUDY OF CROSS-INFECTION IN UROLOGY

BY

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Three hundred and fourteen strains of coliform bacilli (79 *Klebsiella* and 235 *E. coli*), mostly isolated from urine, were investigated for their production of, or sensitivity to, certain colicines.

Only one *Klebsiella* strain showed colicine activity, but in 204 of the *E. coli* strains colicine production or sensitivity was demonstrated.

Colicine typing was of considerable value in epidemiological investigations of post-operative urinary infections due to *E. coli*.

Antibiotic-like substances which are produced by many Gram-negative bacilli and are able to inhibit the growth of related strains were first described by Gratia (1925), and were later called "colicines" (Gratia and Frédéricq, 1946).

Recently, tests based on the ability of bacteria to produce colicines, or on their sensitivity to colicines produced by other strains, have been employed in epidemiological studies of infection by intestinal pathogens, e.g., *Shigella sonnei*, and strains of *Escherichia coli* isolated during outbreaks of gastro-enteritis (Frédéricq, Betz-Bareau, and Nicolle, 1956; Shannon, 1957; Abbott and Shannon, 1958).

This paper describes the use of colicine tests in a study of cross-infection of the urinary tract, mainly among urological patients in this hospital (Miller, Gillespie, Linton, Slade, and Mitchell, 1958).

Methods

The basic techniques were those described by Frédéricq, Thibault, and Gratia (1946) and employed, with some modifications, by Shannon (1957) for colicine-typing strains of *E. coli* which caused gastro-enteritis, and by Abbott and Shannon (1958) for typing strains of *Shigella sonnei*.

From a survey of the properties of colicine production and colicine sensitivity of 200 coliform strains isolated during our studies of urinary infections and of 13 strains obtained by Dr. A. Mayr-Harting (University of Bristol) from Professor P. Frédéricq (University of Liége), standard sets of strains were selected for use in the typing methods described below. Ten colicine-sensitive strains of *E. coli* isolated in this department (nine from urine and one from faeces) were chosen for the colicine production tests and were designated "strain 1, 2 . . . 10." Of the 10 colicine producers used for the sensitivity tests, seven were Professor Frédéricq's strains (Ca 7, Ca 18, Ca 31, Ca 42, Ca 53, Ca 62, K 235); the other three were isolated in this department. The 10 producer strains were designated by the letters "A, B . . . J."

Medium.—Ten per cent. blood agar plates, containing approximately 35 ml. of agar each, were used for all tests. (The agar was deeper than in normal plates to avoid exhaustion of the nutrient; 10% blood was incorporated in the medium as a source of catalase to eliminate inhibition due to any hydrogen peroxide which might be formed during the test (Gardner, 1950).)

(1) **Screening Tests for Detection of Colicine Production.**—Early in the work, it was found that test strains which produced any demonstrable colicines invariably inhibited one particular standard sensitive strain (strain 4). Accordingly, strain 4 was used to provide a screening test for colicine production by unknown test strains. If the screening test was negative, no further production test was done; if positive, the test strain was put up against the full set of standard sensitive strains (see method 2).

The screening test was made by inoculating test strains (six per plate), radiating like the spokes of a wheel, on blood agar plates. The plates were then incubated for 48 hours at 37° C. A disc of filter paper was then placed in the lid of the Petri dish and saturated with chloroform; the dish containing the medium was then replaced and the plate left inverted on the bench for two hours. By this means, the surface layers of the streaks of bacterial growth were killed by the chloroform vapour. Next, the streaks of growth were scraped to the centre of the plate with a clean glass slide and this small area of agar removed. More chloroform was then added to keep the filter paper moist, and the plate left closed for a

further hour. The filter paper was then removed and the plate exposed to the air for two hours, with the lid open and the surface of the medium downwards.

A 1-in-4 dilution of an overnight broth culture of *E. coli* strain 4 (indicator strain) was then prepared and inoculated in a circular streak, crossing the centre of the line of growth of each of the test strains. After incubation overnight, positive results were shown by inhibition of the indicator strain (Fig. 1).

FIG. 1.—Six test strains were grown on each plate in the positions shown by the darkened agar. After treatment with chloroform, the lines of growth were scraped into the centre of the plate and this small area of agar removed. A ring of inoculum (sensitive strain 4) was then added. (*a*) No colicine activity. The smooth and constant growth of sensitive strain produced by the special inoculation wire can be seen. (*b*) Two of the strains produced colicines.

FIG. 2.—Inoculating wire.

In the methods described in the literature, a 3–4 mm. wire loop is used for the inoculation of indicator strains on test plates, but, in our experience, such loops frequently gave irregular and tapering streaks of growth. However, a twisted double platinum wire with the tip bent to serve as a nib, when dipped in the dilution of indicator culture, was found to hold sufficient fluid around the two twisted wires to supply a constant inoculum from the tip over six or seven inches of agar surface (see Figs. 1 and 2). When inoculating a large number of plates, it was unnecessary to flame the wire between each plate, but it was flamed before being introduced into a broth culture of a different organism (Abbott and Shannon, 1958).

(2) **Typing by Colicine Production.**—The coliform strain to be tested (test strain) was inoculated heavily across a plate to give a confluent streak of growth (primary streak). It has been found quite satisfactory to inoculate two test strains per plate. After incubation at 37° C. for 48 hours, the plates were treated with chloroform as previously described for the screening tests, the lines of growth in this case being scraped to one end and removed together with a small piece of agar. After final aeration, the plates were then inoculated with the standard set of colicine-sensitive bacilli (strains 1–10). One-in-four dilutions of overnight broth cultures of these organisms were used, and they were inoculated across the plate at right angles to the line of the primary streak. The plates were incubated at 37° C. overnight, and examined next day.

If no colicine was formed, active against the indicator strains, all streaks grew across the plate as parallel confluent lines of growth. Where detectable colicines were produced, areas of inhibition of one or more of the standard sensitive strains could be seen, distributed symmetrically around the original position of the primary streak (Fig. 3). The inhibition zone sizes were recorded, using the notation described by Abbott and Shannon (1958) as follows:

+++	..	Inhibition zone	..	> 4 mm.
++	..	,, ,,	..	2–4 mm.
+	..	,, ,,	..	<2 mm.
±	..	Thinning only		
−	..	No inhibition		

Subscript R.—Presence of numerous resistant colonies in zone of inhibition

Subscript D.—A form of inhibition consisting of a central area of growth with a zone of inhibition on either side.

FIG. 3.—Three test plates for typing by colicine production.

(3) **Typing by Sensitivity to Colicines.**—The standard colicine-producing strains (A, B . . . J) were grown (two streaks per plate) and treated by the same techniques as the test strains in the production method (2). Overnight broth cultures of the organisms to be tested were then diluted 1 in 4 and inoculated, 10 per plate, on each of the five primarily inoculated plates. Results were normally recorded after 18 hours' incubation using the same notation as for colicine-production typing.

(4) **Controls.**—The standard strains were checked during each series of colicine tests by testing the colicine-producing set of strains against the standard colicine-sensitive set.

Results

Distribution of Colicine-producing and Colicine-sensitive Strains.—Three hundred and fourteen coliform strains, isolated during investigations of urinary infection, have so far been examined by the typing methods described. The organisms were obtained from the following sources: 11 strains from swabs of urethra or perineum, 33 strains from faeces, 269 strains from urine, and one strain from blood.

Seventy-nine of the cultures were members of the genus *Klebsiella*, and only one showed any activity, being colicine sensitive. The remaining 235 organisms were strains of *E. coli*; 97 (41%) produced demonstrable colicines, 172 (73%) showed sensitivity to one or more of the colicines produced by the standard strains, and 31 (13%) showed neither kind of activity (Table I).

TABLE I

COLICINE ACTIVITY OF 235 STRAINS OF *E. COLI*

Number of *E. coli* Cultures	Activity Demonstrated	
	Colicine Production	Colicine Sensitivity
31	−	−
32	+	−
107	−	+
65	+	+
Total 235	97	172

Designation of "Types."—The strains displayed a large number of different colicine patterns, and, in order to record comparisons of strains from different sources, a simple method of designation was adopted for local use. For this, the readings

from colicine test plates were grouped into the following three categories:

Positive	+	Complete inhibition, no account being taken of the size of the inhibition zone or of small numbers of resistant colonies
Doubtful	±	Thinning of growth above the line of the primary streak or a small inhibition zone containing many resistant colonies
Negative	–	Normal growth

The pattern of activity of each test strain was recorded as a series of figures or letters indicating the standard strains against which positive reactions were obtained. Brackets were used when reactions were doubtful.

Examples:

(1) Production typing, *E. coli* (Lab. No. 2004).

	Indicator Strains									
	1	2	3	4	5	6	7	8	9	10
Activity..	+++±	–	++	+++	++±	++±	–	–	–	–
Simplified pattern	+	–	+	+	+	+	–	–	–	–

This pattern was recorded as 1/3/4/5/6.

(2) Sensitivity typing, *E. coli* (Lab. No. 2706).

	Colicine-producing Strains									
	A	B	C	D	E	F	G	H	I	J
Activity..	+++	–	–	–	+±	++	–	–	–	–
Simplified pattern	+	–	–	–	±	+	–	–	–	–

This pattern was recorded as A (E) F.

So far, 30 different patterns of colicine production and 55 different patterns of colicine sensitivity have emerged from these systems of typing.

Most of the patterns were exhibited by only a few strains, but one of the production patterns and one of the sensitivity patterns were frequently encountered; 29 strains gave the production pattern 1/3/4/5/6/8/9/10 and 52 strains had the sensitivity pattern A. The widespread occurrence of these patterns was not thought to be the result of frequent cross-infection by a few established "hospital" organisms, since strains with these patterns were isolated between 1955 and 1959 from both urine and faeces and, during some investigations not reported in this paper, from the faeces of normal persons outside hospital. Moreover, by a variety of methods, comparisons of these strains with those of Professor Frédéricq indicated that organisms of production pattern 1/3/4/5/6/8/9/10 produced colicine V, the most active and common of the colicines, and that organisms of sensitivity pattern A were inhibited only by that strain of the standard set which produced colicine V.

Use of Colicine Typing in Tracing Routes and Sources of Cross-infection.—Colicine typing, by the methods described, has a serious limitation in that it is largely inapplicable to the *Klebsiella* group of organisms which are responsible for much of the cross-infection among urological patients in this as in other hospitals (Ørskov, 1952 and 1954). However, with coliform bacilli other than strains of *Klebsiella*, the methods, by virtue of the high specificity of colicine activity, are very useful in tracing routes and sources of infection.

The property of colicine sensitivity has been found by Abbott and Shannon (1958) to be unreliable as an epidemiological marker in studies of *Shigella sonnei*, but Shannon (1957) has suggested that the unreliability may be due to the production of resistant variants *in vivo* by the action of the colicine-producing organisms which Gratia and Weerts (1946) have shown to be present in almost all specimens of faeces. On the other hand, we have found colicine sensitivity to be a stable and reproducible property of a strain isolated repeatedly from the same patient; but this may be because many of our coliform strains were isolated in pure culture from urine and not from faeces.

Some examples of the use of colicine typing are given below.

(1) On February 26, 1957, Mr. A. L. was admitted from another hospital with an indwelling catheter. The urine was heavily infected with *E. coli* (Lab. No. 3587) and *Streptococcus faecalis*. After operation on March 1, 1957, the patient remained on open drainage (urine draining into an open non-sterile vessel at the bedside) for at least a fortnight. Mr. G. H. was admitted to the next bed on March 4, 1957, and his urine remained sterile until prostatectomy on March 6, 1957, after which an apparatus for closed aseptic drainage and irrigation was fitted (Miller *et al.*, 1958). His urine was sterile next day, but on the same evening the apparatus became disconnected in the ward and was reassembled by the nursing staff, a complication which, experience has shown, usually leads to infection. On March 8, 1957, his urine was found to contain *E. coli* (Lab. No. 3626), and the infection which developed was sufficiently severe to require antibiotic treatment. Unlike any other strains of *E. coli* isolated during the three years of our investigations, both cultures 3587 and 3626 were of the same colicine-sensitivity pattern, A/B/C/(F)/J, and were also identical in biochemical characteristics and antibiotic sensitivity. It is clear, therefore, that Mr. G. H. became infected by the coliform strain which had been introduced into the ward by Mr. A. L.

and had been allowed to proliferate in the urine receptacle near Mr. G. H.'s bed. The infection may have occurred when the closed drainage apparatus was disconnected, probably from the contaminated hands of a nurse.

(2) On January 7, 1958, Mr. P. was cystoscoped in another hospital. His urine contained large numbers of coliform bacilli (Lab. No. 4900). After use, the cystoscope was immersed horizontally in 1/2,000 mercuric oxycyanide solution for 11 minutes before rinsing in sterile water, a procedure which, we have found, cannot be relied upon to disinfect cystoscopes (Miller *et al.*, 1958). The same instrument was then used for the examination of another patient, Mr. C. Coliform bacilli (Lab. No. 4903) were also grown from this patient's urine and from a rectal swab taken at operation (Lab. No. 4907). Specimens of urine were obtained from both patients the following day. Cultures from urine taken during and after operation from Mr. P. showed no change in bacterial flora throughout. Culture of the first urine passed after operation by Mr. C., however, revealed two types of colony; one resembled that grown from his urine before operation, but the other appeared to be different. On the following day, Mr. C.'s urine, on culture, yielded large numbers of the new type of colony, and this organism had biochemical properties identical with those of strain 4900 which had been isolated from the urine of Mr. P. Neither showed any colicine activity, in contrast to the pre-operative urinary and faecal strains of Mr. C. which were clearly different. The results of the colicine tests are given below, and supported the belief that the infection was transferred on an inadequately disinfected cystoscope.

EXAMINATION FOR COLICINE ACTIVITY

		Production	Sensitivity
Mr. P.	Urine 4900	−	−
Mr. C.	Pre-operative urine 4903 ..	−	+
	Faeces 4907 (10 different colonies tested)	+	−
	Post-operative urine 4937 ..	−	−

(3) A urethral swab, taken from Mr. R. C. immediately before undergoing transurethral prostatectomy (November 13, 1957), yielded *E. coli* (Lab. No. 4343). The urine specimens taken before, during, and immediately after operation were sterile, but an infection started on the second post-operative day, caused by *E. coli* (Lab. No. 4351). The infecting strain and the strain isolated from the patient's urethra before operation proved to have identical patterns of colicine production and sensitivity (production pattern 2/3/4/7, sensitivity pattern A). It would appear that small numbers of coliform bacilli, present in the urethra before operation, were introduced into the bladder during instrumentation and subsequently caused urinary infection.

(4) Mrs. S. O. underwent a gynaecological operation on February 5, 1959. Within 18 hours of operation, her urine had become infected by a strain of *E. coli* (Lab. No. 6735), production pattern 1/3/4/5/6/8/(9) and sensitivity pattern B/G/J. A strain with identical patterns was isolated from a rectal swab taken at operation. The infection was probably caused by an organism originating in the patient's own intestine, possibly as a result of contamination during catheterization.

(5) Mrs. R. D. developed a urinary infection after several days of catheter drainage following a gynaecological operation on March 2, 1959. Culture of the urine yielded two different coliform strains. Neither produced colicines, and only one showed sensitivity, giving pattern C/I. Coliform bacilli, clearly different from both of the urinary strains, were isolated from the rectal swab (Lab. No. 6878) taken at operation. These also did not produce colicines but were of sensitivity pattern A. In this case, therefore, the infecting organisms probably came from the environment.

It is a pleasure to express my gratitude to Dr. W. A. Gillespie for his help and encouragement, and to the Board of Governors of the United Bristol Hospitals for a grant towards the cost of this research.

I would also like to thank Dr. A. Mayr-Harting and Professor P. Frédéricq for some cultures, and Dr. M. T. Parker, Dr. J. D. Abbott, and Mr. R. Shannon for teaching me their techniques of colicine typing.

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TECHNICAL METHODS

A Technique of Blood *p*H Estimation

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The blood *p*H can be measured electrometrically by means of a glass electrode and calomel reference electrode provided (*a*) the blood is kept from contact with air and (*b*) the measurement is made at 38° C. (Wynn and Ludbrook, 1957). Alternatively to (*b*), but less satisfactorily, the *p*H can be estimated at an arbitrary temperature (say 20° C.) and a correcting factor employed.

To satisfy the above requirements a closed circuit of small total volume may be used. The apparatus shown here has the advantage that it can be assembled from standard equipment in any department which has access to adequate glass-blowing facilities. The method can be used for clinical determination of blood *p*H; it is well suited also for performing repeated estimations in small animals, such as the rabbit, without depleting the circulation, since each sample can be re-injected after use.

An exploded sketch is shown in Fig. 1 and a photograph of the assembled apparatus in Fig. 2. A 1 mm. bore glass capillary tube is bent to a flattened U so that it can be suspended by means of a suitable holder in a water-bath at the desired constant temperature. Two small chambers with lateral ports are made, one for the glass electrode and the other for the electrolyte junction to the calomel half-cell. To avoid expensive ground-glass joints these components are fitted into their respective ports by means of sleeves cut from P.V.C. tubing. A commercially available miniature glass electrode of standard pattern is used.

One difficulty was to devise an electrolyte junction which could be made easily and cheaply and which would stand the pressures caused by re-injection of blood against arterial resistance. These requirements are met by packing the lumen of a glass tube very tightly with chemically clean glass-blower's (asbestos) wool impregnated with agar gel embodying 3.5 M KCl.

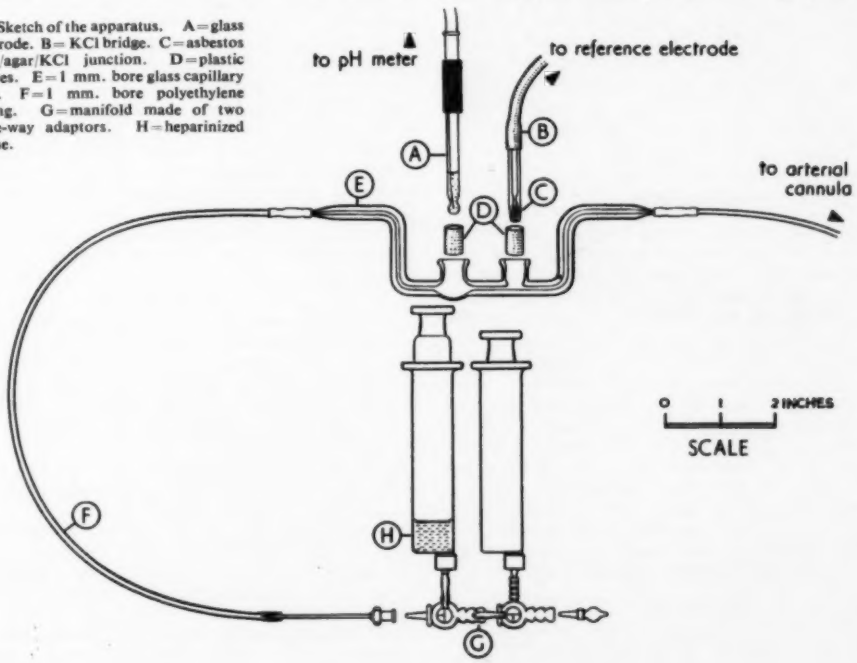

FIG. 1.—Sketch of the apparatus. A = glass electrode. B = KCl bridge. C = asbestos fibre/agar/KCl junction. D = plastic sleeves. E = 1 mm. bore glass capillary tube. F = 1 mm. bore polyethylene tubing. G = manifold made of two three-way adaptors. H = heparinized saline.

0°

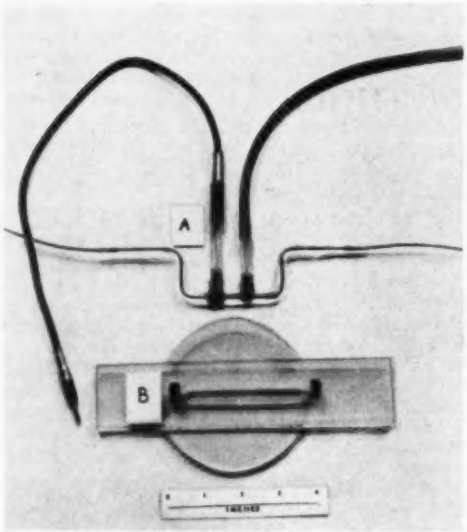

FIG. 2.—A = blood *p*H assembly. B = " perspex " holder.

Tight packing is achieved by ramming the asbestos very firmly against a constriction made at a suitable level in the tube. The agar gel minimizes the packing being soaked with blood. Several of these junctions are kept in a jar of 3.5 M KCl, and the one in use is changed for each series of estimations; after use it is washed with distilled water and returned to the jar of KCl. Electrolytic continuity with the calomel electrode assembly is by a P.V.C. tube containing 3.5 M KCl.

Method of Use

Polyethylene tubes of 1 mm. bore are attached to each end of the apparatus and the system is filled with heparinized saline. For use in the experimental animal the femoral artery is cannulated with similar tubing, which can be joined to one tube of the apparatus by an appropriately sized hollow needle with the butt removed. The other tube of the apparatus is connected by way of a needle to a manifold constructed of two three-way adaptors (see Fig. 1); two 10 ml. syringes, one containing heparinized saline, are attached. By appropriate manipulations the saline filling the system can be drawn into the first syringe, followed by arterial blood into the second syringe. Suitable tilting of the apparatus helps these manœuvres and allows the chambers to be completely and uniformly filled with fresh arterial blood. By reversing the actions the blood can be re-injected and the apparatus left full of heparinized saline. With repeated estimations, of course, there is some loss of blood into the first syringe, and some saline enters the circulation. Since the total volume of the system (including the polyethylene tubing) is only 1.5 ml., however, this interchange is not large. The apparatus can be disconnected from the animal and washed out at any stage of the experiment.

Precautions and Results

With certain precautions, consistent reproducible results have been obtained within the limits of performance of the *p*H meter used.

Buffers used to standardize the meter must be washed out of the apparatus with great thoroughness. Air bubbles must be trapped or evacuated. During the actual reading the arterial cannula should be clamped and disconnected temporarily from the apparatus. If this is not done interference may occur, even when the animal is earthed. Before each reading, the sample should be allowed to stand in the apparatus for about five minutes, to permit equilibration of the temperature of the blood with that of the water-bath. In practice this can be checked by ensuring that the reading has reached a sustained stable level.

For clinical estimation of blood *p*H, the sample is taken in a lubricated heparinized syringe which is then sealed. The estimation is made very simply by filling the empty apparatus slowly from the syringe, tilting it during filling so as to avoid trapping air in the chambers.

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A Capillary Tube Method of Rh Typing Using Albumin Agglutinating Anti-D Serum

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(RECEIVED FOR PUBLICATION JULY 10, 1959)

The capillary tube technique of Rh typing (Chown, 1944; Chown and Lewis, 1946) is a very useful rapid method which, however, depends on using sufficiently potent saline agglutinating anti-D serum, the supply of which is restricted. A method using Chown's technique with albumin agglutinating anti-D serum diluted with 20% bovine albumin has been described (Tuck, 1951), but it has been found that the method described below, which differs from Chown's technique in a number of points, and uses 30% bovine albumin, gives more conclusive results. The albumin agglutinating anti-D serum need not be especially potent; that used for routine tube testing has been found to be satisfactory. The method also has the advantage that no special preparation of serum, test cells, or bovine albumin is necessary.

Method

The test is performed in capillary tubes 90 mm. × 1 mm. (lymph tubes supplied by Baird and Tatlock have been found satisfactory). Small batches of these tubes should be placed in test-tubes, which are then plugged with cotton wool, and sterilized in the hot-air oven before use.

The anti-D serum is probably best kept in 1 ml. quantities in tubes, rather than in bulk in bottles, and care should be taken to avoid handling the end of the capillary tube which is dipped into the serum, in order to minimize any risk of contamination.

The specimen of clotted blood is shaken so that a 25%–50% suspension of cells in serum is obtained. If necessary the cell serum suspension must be centrifuged and the serum decanted in order to obtain a cell suspension of sufficient strength.

The capillary tube should be held horizontally throughout the filling process. The end of the tube is first dipped into the anti-D serum until 1.5 to 2 cm. is filled with the serum. After wiping any excess serum off the outside of the tube on the edge of a piece of blotting paper, the same end of the tube is dipped into the cell serum suspension, and an amount equal to that of the anti-D serum is allowed to enter. Care should be taken to avoid small clots entering, or air locks between the cells and serum. After again wiping the outside of the tube on the edge of the blotting paper to remove any cells, the same end is dipped into the 30% bovine albumin, and an amount equal to the combined volume of cells and anti-D serum is allowed to enter, once more avoiding air locks between the albumin and the cells and serum. Having wiped any excess albumin off the outside of the end of the tube, still holding the tube horizontally, the end opposite to that used for filling the tube is gently inserted into a block of "plasticine," and, as soon as the end is blocked, the tube is elevated into the vertical position, so that the anti-D serum is below, the cells are above this, and the bovine albumin is uppermost.

The test is placed in a 37° C. incubator and in most cases the result can be read after five minutes or less, and in all cases a final result can be read in 10 to 15 minutes.

The tests may be read with the naked eye, or with the assistance of a low-power lens in a good light against a white background. Rh-positive tests show large clumps of agglutinated cells extending down the tube; in Rh-negative tests the cells extend down the tube in a slightly ropy but continuous column, which tends to remain distributed throughout the length of the tube, in marked contrast to the positive tests, where the clumps of agglutinated cells settle rapidly into the lower half of the tube. If the tests are allowed to stand on the bench for an hour after being read, the contrast between positives and negatives becomes even more marked, as in positives the agglutinated clumps pack down to the bottom of the tube, whereas in negatives the cells still remain distributed in a smooth suspension.

If a test is observed with a lens immediately after setting up, it will be seen that the albumin, being heavier than either the cells or serum, descends rapidly, pushing the cells into the serum, and these, having mixed, rapidly ascend to occupy the upper part of the tube, while the albumin takes up the lower position (this was actually shown to happen by carrying out tests with dyed albumin). Next, if the cells are Rh positive they are agglutinated, and the cells go into clumps which fall downwards into the lower part of the tube. If, however, the cells are Rh negative, they are not agglutinated, and descend more slowly.

The above method has been used in testing over 1,000 Rh groups in parallel with a standard method of Rh typing; and in no case has a test been read as positive which in fact proved negative by the standard technique. In three tests a negative result was read when the tests were actually positive by the standard method, but in each case the cell suspension was too weak, and the tests proved positive on being repeated with a stronger cell suspension. A number of D^us were encountered in the series, and all gave a negative result with the test.

It should be emphasized that the test as described is primarily a D screening test, and all specimens giving a negative result must be further investigated by the recommended standard techniques.

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A Modified Quick's One-stage Test for the Control of Out-patients on Anticoagulant Therapy

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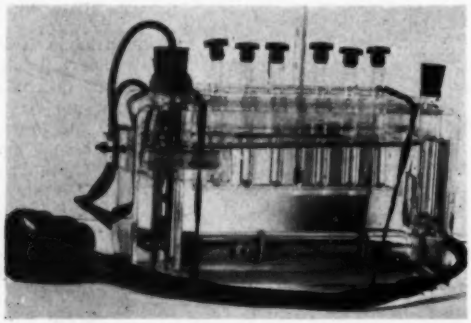
(RECEIVED FOR PUBLICATION NOVEMBER 5, 1959)

In Quick's one-stage test, stage 1 is probably "short-circuited," tissue thromboplastin taking over the function of blood thromboplastin. As a consequence the modified test consists simply of performing the test on fresh whole blood instead of on plasma. The advantage is particularly applicable to out-patients, as the prothrombin time can be estimated straight away and the follow-up dose of the anticoagulant drug prescribed there and then. In a controlled series of 200 tests run in parallel with the standard Quick's method, the incidence of variation of more than two seconds is about 1.2%.

Method

It is essential to have a 37° C. water-bath close at hand, so that the venepuncture specimen does not lose too much heat. A small portable electric water-bath (37° C.) is ideal for this purpose (Fig. 1).



Approximately 0.5 ml. of blood is taken from a vein, and, without delay and with the needle still *in situ*, 0.2 ml. of the specimen is delivered into each of two 3 × ½ in. tubes (pre-heated). The water-bath is fitted with a 0.2 ml. line for convenience. At least a minute is then allowed for the specimen to reach 37° C., and 0.2 ml. of brain thromboplastin (in a pre-heated tube) is added to the first tube, and the time determined for clotting. The end-point is easier to determine if the tubes are plugged and then gently rocked over an opaque light source. When a clot forms and the specimen ceases to move is the end-point. The test is repeated on the second tube, and the average of the two readings taken. This time is the prothrombin time of the sample.

It is advisable, in colder weather, to use pre-heated (37° C.) syringes and needles to take the blood specimen.

A Manometric Micro-apparatus for Estimating Oxygen

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(RECEIVED FOR PUBLICATION AUGUST 27, 1959)

In previous communications (Rappaport, Eichhorn, and Nutman, 1956a and b) an apparatus for manometric determination of CO_2 in 0.1 ml. and 0.05 ml. of plasma was described. The problem of examining oxygen in the same apparatus was still unsolved, but now a method for oxygen determination in 0.1 ml. blood samples using the same apparatus has been devised.

Certain precautions must be observed in constructing the apparatus (Fig. 1). Stopcock A should have a capillary bore not more than 1 mm. This is important when introducing sodium hydroxide for CO_2 absorption, so that NaOH enters easily without mixing with hydrargyrum, which is used as a seal immediately afterwards. If the bore is wider, the remains of NaOH, together with the mercury inside, hinder a constant reading as NaOH continues to flow inside the chamber. The neck of cup M should also be exactly formed. It should be short enough to allow the micro Oswald pipette to touch the opening of the stopcock when introducing the blood into the extraction chamber and also facilitate the sealing with Hg. Each drop of Hg should fall directly on the bottom of the neck, without air or fluid bubbles. The jacket of the chamber (Fig. 2) should be attached in such a way that the 0.1 ml. mark of the reaction chamber should be easily legible.

The micro Oswald pipette should preferably be made out of a part of a 0.2 ml. pipette graduated in 0.001 ml. so that any quantity of blood introduced into the chamber can be easily calculated. For example, if 0.105 ml. blood is introduced by mistake, the calculation can easily be corrected and the examination need not be repeated.

Reagents

(1) Extraction Solution (Modified) (Rappaport and Köck-Molnar, 1934).

A. 125 g. urea
2 g. saponin alb. Merck
1.5 ml. octyl alcohol
3 ml. concentrated lactic acid
125 ml. distilled water

B. 28% potassium ferricyanide kept on ice

* The work was completed before Professor Rappaport's illness and subsequent death on July 17, 1959.

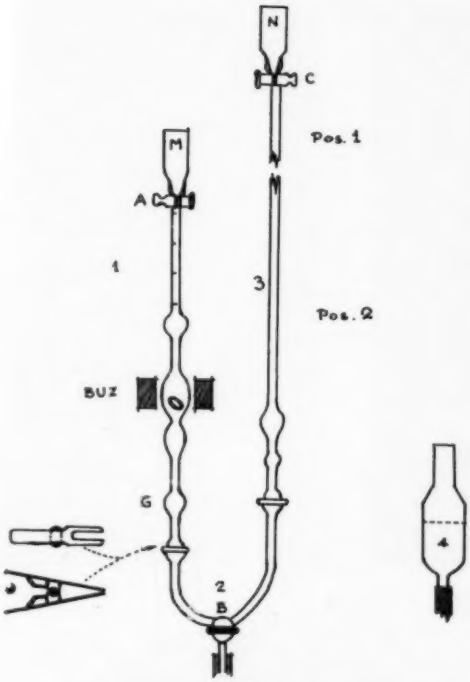

FIG. 1

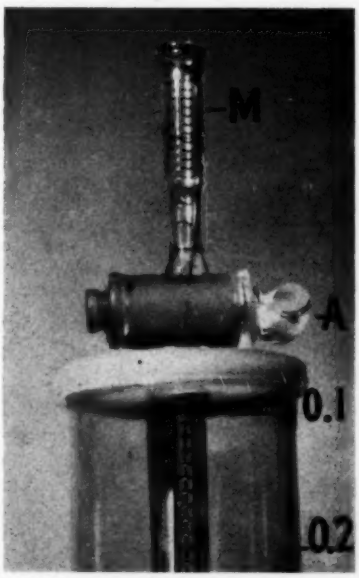

FIG. 2

Mix 2 ml. of A and 0.5 ml. of B just before use. In this solution the octyl alcohol is dissolved homogenously and does not form an upper layer. With its higher urea content, it prevents the coagulation of proteins and keeps the apparatus clean. It is advisable to use this solution also in the macro Van Slyke apparatus (Rappaport and Köck-Molnar, 1934). Because of the high specific gravity of this solution, the blood introduced into the chamber should run in directly, otherwise it will be dispersed and flood into the extraction solution in the cup. If the construction of the cup M has not been entirely satisfactory the following solution of a lighter specific gravity must be used.

Alternative Extraction Solution (Van Slyke and Neill, 1924; Barcroft, 1914).

C. 2 g. saponin
3 ml. lactic acid
1.5 ml. octyl alcohol
To 250 ml. distilled water

Mix 2 ml. of C with 0.5 ml. of B just before use. Urea is omitted and therefore the beneficial influence of hindering protein precipitation is missing.

The apparatus must therefore be cleaned after each examination with solution 4. The ferricyanide (B above) if kept separately in the refrigerator is stable for a long time.

(2) 2 N NaOH.—This is made gas free, preferably in a macro Van Slyke apparatus, and kept under paraffin oil in a calcium chloride tube reservoir as described by Van Slyke. The end of this tube should be equipped with a polyethylene tube (Rappaport and Eichhorn, 1955) and 0.1 ml. of the NaOH solution should give a strong alkaline reaction with phenophthalein when 0.3 ml. of the extraction solution is added.

The apparatus is calibrated as described for CO_2 estimation (Rappaport *et al.*, 1956b). An apparatus which has been calibrated for CO_2 can be used for oxygen without any further preparation. The micro apparatus built by Gallenkamp is calibrated in such a way that the 0.1 and 0.2 ml. marks correspond exactly to these amounts. Apparatus built in the laboratory should be carefully calibrated based on examinations in a macro Van Slyke apparatus.

Procedure

(1) Wash the apparatus with about 0.5 ml. octyl alcohol to free it from all fat particles, otherwise the introduction of NaOH is difficult. This is done once before each series of examinations.

(2) Introduce about 1 ml. of extraction solution and free from gas by shaking for two minutes.

(3) Expel gas and raise the fluid into the cup till a small quantity of mercury appears. Introduce 0.1 ml. of blood with a micro Oswald pipette tipped with polyethylene (Rappaport and Eichhorn, 1955) and follow it with the extraction fluid till mark 0.4 ml. is reached. (The levelling bulb should be in position 2, as at this position the fluid can

enter the reaction chamber capillary only down to the 0.4 ml. mark.) Seal with mercury, fill the bore of stopcock A with mercury and wash cup M with distilled water. Lower the mercury inside just below the reaction chamber proper (E), where the buzzer stirrer is enclosed, and shake for two minutes (the mercury should not be touched by the stirrer so that it remains clean). Raise the fluid slowly till mark 0.2 ml. and read p1. Then raise the fluid to the maximum (levelling bulb in position 2 parallel to 0.4 ml. mark) and introduce 0.1 ml. of 2 N NaOH very carefully, seal with mercury, including the boring of stopcock A, and wash the cup with distilled water. Read the pressure on the manometer when the fluid meniscus is on the 0.2 ml. mark (p2). Read again on the 0.1 ml. mark (p3). Expel air and 0.1 ml. fluid. Seal with mercury and read p4 on the 0.2 ml. and p5 on the 0.1 ml. mark.

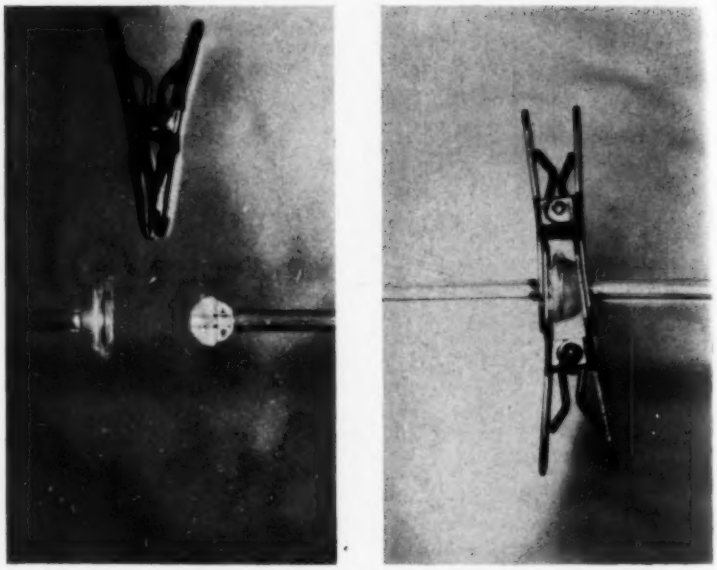

FIG. 3

Calculation

This is done according to the table of Van Slyke and Neill (1924). For CO_2 content, p1–p2 is multiplied by the factor found under CO_2 content, namely,

volume=1 ml., a=2 ml.

For oxygen content, p2–p4 is multiplied by the factor found in the same table under oxygen content, namely:

volume=1 ml., a=2 ml.

The result gives the CO_2 and oxygen contents respectively in volume per cent.

For example, temperature=20° C., factor for CO_2=0.2655, and factor for O_2=0.2450. p3–p5 multiplied by the factor and divided by 2 give the same oxygen content (the reading is only higher, which is preferable for small quantities).

If the CO_2 content is of no interest, as in heart catheterization where oxygen and oxygen capacity only are important, the reading of p1 can be omitted. For this purpose, attempts were made to extract the oxygen directly in an alkaline medium, as in the procedure of Barcroft (1914) and Haldane with their volumetric apparatus, but they failed. Apparently mercury or octyl alcohol interferes with the process and the results were constantly too low.

The results for CO_2 and oxygen obtained in the micro apparatus compared favourably with those of the original Van Slyke apparatus.

To simplify the apparatus further, absolute airtightness was achieved by using simple ball joints with two clamps (Fig. 3), and not as described above with a mercury sealing. When the apparatus has to be cleaned and the ball joints are frozen, they can easily be freed by warming with a hair dryer.

Gallenkamp has constructed a macro apparatus with ball joints; a micro attachment for it was made by removing the extraction chamber and replacing it with a micro one as described above. The combined apparatus gave very satisfactory results.

Summary

An improved extraction fluid, precautions to be taken in constructing the micro apparatus, and the procedure for the determination of the oxygen content of blood in 0.1 ml. samples are described, using the manometric micro apparatus for gas analysis.

It is also possible to use the same micro extraction chamber and attach it to a macro apparatus, provided the same size of ball joints is used for both types of apparatus.

We are grateful to Mrs. Jehudith Shashdy for her assistance in performing the final experiments.

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An Orbital Shaker

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Conventional shaking devices of the reciprocating pattern are frequently noisy, mechanically complex, and may consume considerable power for the work they are required to do. Various attempts to reduce some or all of these disadvantages have resulted in machines such as the suspended shaker devised by Kantovitch ; but this, like most if not all others, is of a reciprocating pattern.

A laboratory shaker, which could be left running continuously and reliably for long periods in a room where others were working, was required, and an orbital shaker has been developed (Fig. 1) which does not possess the disadvantages of reciprocating shakers. The prototype is suitable for the continuous shaking of 16 flasks of 250 ml. capacity each containing about 150 ml. of fluid.

The shaking unit consists of a number of tiers (in the prototype, two, but there is no reason why more should not be used), on which the flasks are arranged in a regular pattern. Through the centres of the platforms constituting the tiers passes a shaft running in ball bearings top and bottom. Two arms carry out-of-balance weights, adjustable in position and mass, at each end of the shaft. When the shaft is rotated an orbital shaking motion is transmitted to the unit which is suspended in an angle iron frame by wire or nylon cords. The amplitude of the shake is determined by the position and size of the weights, whilst the frequency of the shake is determined by the shaft speed. The shaft is connected by a flexible coupling to a fractional horse-power motor fitted with a speed control. To obtain complete stability, the motion was damped by suitable lengths of flexible tubing connecting the frame to the shaking unit.

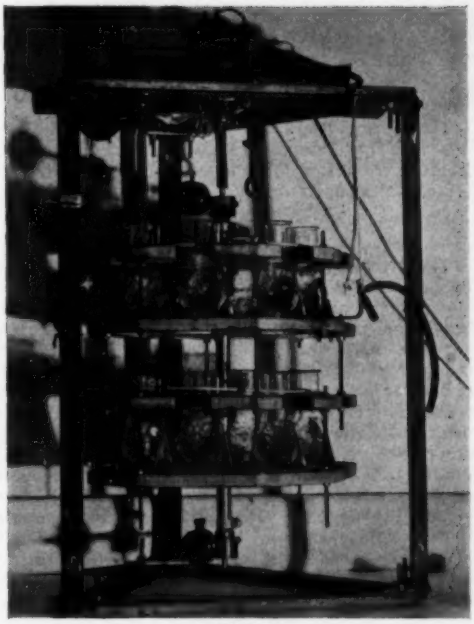

FIG. 1

The shaker has been left running unattended for long periods and is exceptionally quiet in operation.

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Seventh European Congress of Haematology

The Seventh European Congress of Haematology was held in London under the presidency of Dr. John F. Wilkinson, Manchester. Brief summaries of some of the papers follow.

Erythropoiesis

The development of pernicious anaemia was traced by L. J. WITTS (Oxford) mostly to anomalies in the stomach; he pointed out that achlorhydria plays an important part in the production of pernicious anaemia, and stressed the aetiological factors of inheritance. The pathology of the stomach in pernicious anaemia was outlined by H. A. MAGNUS (London). E. LESTER SMITH (Greenford, Middx.) described some of the anti-vitamin B_{12} substances. He pointed out that vitamin B_{12} has not yet been synthesized, but modification of the nucleotide part of the molecule produces analogues which have some vitamin B_{12} activity. The replacement of ammonia in one of the side-chains by methylamine and similar amines, or other bases, produces analogues with an antimetabolite activity. The anti-vitamin B_{12} activity of the substances can be estimated by tests involving bacterial mutants and the growth rate of chicks. M. SCHWARTZ and P. LOUS (Copenhagen, Denmark) pointed out that in the treatment of pernicious anaemia, oral preparations of vitamin B_{12} and pyloric mucosa often failed after a good early response due to resistance to the heterologous intrinsic factor. An inhibiting substance is sometimes present in the serum of patients with pernicious anaemia, but on other occasions there may be intestinal blockage.

Folic acid deficiency was investigated by R. H. GIRDWOOD (Edinburgh), who used folic acid absorption tests and investigated the rise of serum folic acid after a test dose and the urinary contents of glutamic acid. He pointed out that folic acid deficiency may occur in nutritional megaloblastic anaemia, steatorrhoea, after partial gastrectomy, in the presence of blind loops of the intestine, in pregnancy, and in chronic liver disease. The diagnosis of folic-acid deficiency was outlined by I. CHANARIN (London), who described the sequence of the disposal of parenteral doses of folic acid by measuring the excretion of folic acid in the urine and the rate of clearance from the plasma to the tissues.

Sideroblastic anaemia was discussed by L. HEILMEYER (Freiburg, Germany). He grouped together idiopathic, sideroblastic anaemia, thalassaemia, as well as an inherited hypochromic anaemia which does not respond to iron, cobalt, and pyridoxine, and the sideroblastic refractory anaemia which is sometimes accompanied by leucopenia. He assumed that these anaemias were due to an enzyme deficiency. M. BESSIS (Paris, France) described his electronic microscope studies in sideroblastic anaemia. Granules of sideroblasts show different aspects: ferritine molecules may be surrounded by a membrane; mitochondria may be filled with ferritine or they may be filled by other iron fragments more or less mixed with ferritine. At present no distinction can be made between the various sideroblastic anaemias.

C. A. FINCH (Seattle) discussed the plasma iron turnover, particularly between the tissues ejecting iron and the plasma. The size of the iron turnover largely depends on the capacity of the erythropoietic marrow to take up iron and therefore is a measure of the activity of the erythroid marrow. L. HALLBERG (Göteborg, Sweden) used a double radio-isotope technique in order to study iron absorption. It was possible to estimate the outflow of iron from the plasma and to calculate also the rate of absorption. The relation between the rate of iron absorption and the erythropoietic marrow activity is effected by the plasma iron-transferrin system. There is no evidence of mucosal blockage of iron absorption. After partial gastrectomy, iron-deficiency anaemia is very common. J. R. HOBBS and G. DISCOMBE (London) found that the anaemia was determined by bleeding from some other side, or failure to treat iron depletion, or failure of iron absorption.

C. A. A. SCHRUMPF (Porsgrunn, Norway) related his experiences with vitamin B_{12} assay in pernicious anaemia. He stressed the importance of prepernicious states after gastrectomy and in sideropenia. A. DOSCHERHOLMEN, P. FINLAY, and P. S. HAGEN (Minneapolis, Minn., U.S.A.) estimated the organ distribution of radioactivity in man after the ingestion of radio-labelled vitamin B_{12}. They found that vitamin B_{12} moves in and out of several different body compartments: (1) a temporary storage compartment, i.e., the wall of the small intestine; (2) a transport compartment, i.e., the blood; (3) a permanent storage compartment, i.e., the liver.

J. Q. MATTHIAS (London) studied the survival of red cells in several types of proliferative diseases of unknown aetiology by means of radioactive chromium. Survival was related to fever, size of liver and spleen, degree of anaemia, marrow involvement, and several other tests. L. K. DIAMOND and T. SHAHIDI (Boston, Mass., U.S.A.) found that in some cases of aplastic anaemia after much ill success with many other methods of treatment testosterone produced a remission in eight patients when it was combined with corticosteroids.

Myelopoiesis

In a session on aetiological and experimental aspects of leukaemia presided over by A. Haddow (London), J. ENGELBRETH-HOLM (Copenhagen, Denmark) emphasized the changing pathological aspects of leukaemia, pointing out that longer survival gives rise

to changed morbid anatomical patterns. The role of the thymus in murine lymphomas was discussed by J. F. A. P. MILLER (London). He inoculated cell-free filtrate extracts of leukaemic tissues intraperitoneally into newborn and adult mice. Total thymectomy completely prevents the development of leukaemia in mice susceptible to leukaemogenic activity of the filtrates, but thymus grafting restores its potentiality. J. M. YOFFEY (Bristol) studied the lymphocytes in the bone marrow of the guinea-pig and found no lymphopoietic foci in normal marrow and very few lymphocytes in mitosis. He suggested that the micromyeloblasts and the primitive cells of Cunningham, Doan, and Sabin are probably small lymphocytes. He found that after sublethal irradiation the number of lymphocytes in the marrow increases about two-and-a-half-fold and then decreases when the red and white cell marrow portions become more active.

The differential diagnosis of monocytic leukaemia was outlined by H. STOBBE (Berlin, Germany), who recognized Schilling and Naegeli types. He suggested that phase-contrast microscopy of living preparations may sometimes contribute to a solution of such diagnostic programmes.

In a session devoted to morphology of leucocytes, E. UNDRITZ (Basel, Switzerland) outlined the hereditary constitutional anomalies of leucocytes and the abnormalities in shape and structure of nucleus or cytoplasm. Leucocyte anomalies are independent of race and sex. Amongst the dominant hereditary anomalies, the best known of the nuclear anomalies is the Pelger-Huët anomaly, and, amongst the cytoplasmic anomalies, the May-Hegglin anomaly. Amongst the recessive hereditary anomalies, he listed Alder's anomaly. The culture of bone marrow in acute leukaemia was illustrated by a film shown by R. J. V. PULVERTAFT and J. G. HUMBLE (London).

H. RIEDER (Vienna, Austria) found that patients treated for many years with "myleran" sometimes developed unpleasant side-effects such as pigmentation of the skin which, at biopsy, resembles the findings in Addison's disease, and secondary myelosclerosis, which may occasionally completely mask the appearance of myeloid leukaemia. Mannomustin and related sugar compounds had good clinical effects on certain lymphomas, but C. SELLEI (Budapest, Hungary) was disappointed in its effect on leukaemias.

Using a skin window technique, P. RIIS (Glostrup, Denmark) investigated the relationship between free inflammatory cells in leukaemia and leucocytes in the blood. He never found leukaemic cells in artificial blisters.

In a session on myeloproliferative syndromes, I. A. KASSIRSKY (Moscow, U.S.S.R.) discussed the clinical picture of osteomyelo-reticulosis, which he regards as an atypical leukaemic process. He found treatment with corticosteroids and "myleran" occasionally helpful.

In a session devoted to abnormalities of globulins, R. DI GUGLIELMO (Florence, Italy) found an increase of serum mucopolysaccharides in Waldenström's disease. J. R. SQUIRE (Birmingham) reported on the 61 patients observed by the Medical Research Council Working Party on Hypogammaglobulinaemia. In this condition upper respiratory and pulmonary infections predominate. The incidence amongst male children is about 1 per 100,000 births. The disease is regarded as a low production syndrome. This was established by investigations with radioactive-iodine-labelled proteins.

Coagulation and its Disorders

In a symposium on the clinical use of antihaemophilic globulin, M. BLOMBÄCK and B. BLOMBÄCK (Stockholm, Sweden) outlined the preparation of antihaemophilic globulin (A.H.G.). I. M. NILSSON (Malmö, Sweden) discussed the use of human A.H.G. in haemophilia A and in von Willebrand's disease. Fraction I–O was given to 24 haemophilic patients with very good clinical improvement. No resistance was observed. The plasma A.H.G. levels were increased from 40 to 80% of the normal before operations and maintained afterwards at 20 to 30%. In cases of von Willebrand's disease purified A.H.G. helped to produce considerable improvement. A.H.G. is not identical with fraction I–O. P. WOLF (Lewisham) described his experience in treating 25 haemophilic patients for troubles with joints, and their prevention; 60% of large tissue and joint haemorrhages responded to rest alone; only in 9% of cases treated with human A.H.G. were there some reactions. F. NOUR-ELDIN (Manchester) discussed the importance of the Bridge anticoagulant in patients to whom A.H.G. had been given. R. BIGGS (Oxford) reported experiences with animal A.H.G. It was found that A.H.G. levels of 5% may be sufficient to arrest bleeding in a severely affected patient. Sometimes levels of 25% are necessary.

In a symposium on long-term anticoagulant therapy, P. A. OWREN (Oslo, Norway) described the laboratory and social organization of long-term anticoagulant therapy in Norway. Standardized reagents are prepared at the coagulation laboratory and distributed to all hospitals. The thrombotest is used extensively. It has been found simple and useful and this has led to a great increase in anticoagulant therapy. I. S. WRIGHT, E. MCDEVITT, and J. S. LIEBERMAN (New York, U.S.A.), M. TOOHEY (London), and A. S. DOUGLAS (Glasgow) discussed various aspects of the problem of long-term anticoagulant therapy.

A. AAS (Oslo, Norway) described the technique of labelling platelets with radioactive chromium. He found that human platelets lived for 9 to 11 days. In idiopathic thrombocytopenic purpura normal platelets survive for only a few hours, but in aplastic anaemia they have a normal survival time. J. H. LEWIS and I. SZETO (Pittsburgh, Penn., U.S.A.) made observations on normal human platelets and found that the circulating platelet radioactivity levels reach a maximum of 2 to 24 hours.

The biological equivalents of blood coagulation factors were described by P. DE NICOLA (Pavia, Italy). His discussion concerned phospholipid enzymes, Russell viper venom, and physiological fluids such as bile, saliva, and seminal fluid. The genetic aspects of factor VII deficiency were discussed by F. E. DISCHE (London), who had observed a family, including a woman, with mild haemorrhagic diathesis. G. I. C. INGRAM (London) reported his work on the Bridge anticoagulant.

The interrelationships of vitamin K_1 and of anticoagulants in the synthesis of prothrombin were discussed by S. A. JOHNSON and E. MCCALL PRIEST (Detroit, Michigan, U.S.A.), and H. B. ANSTALL (London) provided evidence that there may be a substance activating Christmas factor which may be distinct from either the Hageman factor or plasma thromboplastin antecedent (P.T.A.). The role of magnesium on the clotting of human blood was discussed by H. B. ANSTALL, R. G. HUNTSMAN, B. HURN, and H. LEHMANN (London).

L. B. JAQUES and G. A. MCMURRAY (Saskatoon, Canada) described capillary resistance and blood pressure changes associated with pain due to local cooling (the cold pressor test).

Bone Marrow Transplantation

In a session on bone marrow transplantation, J. F. LOUTIT (Harwell, Berks) outlined the process of haemopoiesis after bone marrow transplantation. D. W. VAN BEKKUM (Rijswijk, Holland) discussed the factors influencing the take and the rejection of bone marrow grafts. These factors are antigenic differences between host and donor, the dose of whole body irradiation administered to the host, the time interval between irradiation and transplantation, and the transplantation technique, including the number of donor cells. Marrow transfusion in man was described by G. MATHÉ and J. BERNARD (Paris, France), who stressed the importance of the secondary phenomenon, which usually occurs between 45 and 90 days after irradiation. H. E. M. KAY and M. CONSTANTOULAKIS (London) sketched the problems of storage and the use of foetal cells. The effect of 6-mercaptopurine on transplantation immunity was discussed by R. SCHWARTZ (Boston, Mass., U.S.A.), who found that these effects are somewhat similar to those found in irradiated cases. Much discussion followed these papers, but it was felt that bone marrow transplantation is by no means fully ready for clinical experiments.

Haemolysis and Haemoglobinopathies

In a session on genetically determined enzyme defects of erythrocytes, presided over by J. V. Dacie (London), G. SANSONE (Genoa, Italy) discussed favism and allied haemolytic anaemias. Favism causes diminution and marked instability of the blood glutathione, a tendency to form Heinz bodies, and a deficiency of glucose-6 P-dehydrogenase. T. A. J. PRANKERD (London) discussed enzyme defects in hereditary spherocytosis.

V. M. INGRAM and H. LEHMANN (Cambridge, Mass., U.S.A., and London) described chemical studies on haemoglobin A and S and the difference in their amino-acid composition which often involved a charged carboxyl group; this may explain the difference in electrophoretic behaviour. S. CALLENDER (Oxford) described her genetic and clinical studies on thalassaemia in Britain.

A second haemoglobin abnormality in haemoglobin H disease, involving a "fast" haemoglobin fraction on starch-gel electrophoresis, was described by P. FESSAS (Athens, Greece). L. ZANNOS-MARIOLEA and P. CHIOTAKIS (Athens, Greece) had investigated 18 families with favism and found that the defect was sex linked. J. V. GARRETT and F. MORTON (Birmingham) described an English family with the thalassaemia trait.

Blood Transfusion

In a symposium on the detection and significance of weakly reacting antibodies arranged in conjunction with the International Society of Blood Transfusion, presided over by P. L. Mollison (London), several papers concerned the study of chromium-labelled red cells, the Coombs test, antihuman globulin sera and non-gamma-globulin reagents.

In a session on blood group substances, S. D. LAWLER (London) described blood group substances in human milk, blood, and saliva. A. E. SZULMAN (Boston, Mass., U.S.A.) located human blood group antigens A and B by fluorescein-labelled human hyperimmune antisera applied to frozen sections of surgical and necropsy material.

In all the sessions lively discussion followed the papers. It was felt that these discussions and their continuation, even during the social events connected with the Congress, helped to bring together haematologists from all corners of the world and enabled them to understand each other's problems, as well as bringing them together as friends and making bibliography come to life.

VIIIth Colloquium on Protides of Biological Fluids

The VIIIth colloquium on "Protides of Biological Fluids" will be held from May 6–8, 1960, at St. Jans Hospital, Bruges. For all information please apply to Dr. H. Peeters, The Laboratory, St. Jans Hospital, Bruges, Belgium.

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BOOK REVIEWS

The Pathology of Tumours of the Nervous System. By Dorothy S. Russell and L. J. Rubinstein. (Pp. 318; 286 figures. 70s.) London: Edward Arnold. 1959.

Just before the last war a number of colleagues of the late Dr. J. G. Greenfield were encouraging him to write a textbook on cerebral tumours as well as on neuropathology. He wrote the latter, which has recently been published. Dr. Greenfield felt unable to accomplish the former and so passed this onerous task to Professor Russell. She and Dr. Rubinstein have drawn upon their very wide experience, together with an all-embracing survey of the literature, and have given to pathologists a volume that will surely remain the standard work on the pathology of cerebral tumours for very many years to come. Almost every pathologist at some time has the task of reporting on the histological nature of intracranial or intraspinal tumours, and all will be indebted to these two authors for their extremely lucid description of all varieties of such tumours.

The chapters deal with the varying types of tumour that may be found, ranging from those of a maldevelopmental nature, the retina, and peripheral tumours of the neurone series, including phaeochromocytomas. In addition to the more common types of tumour, such as the glioma and meningioma, there is of course a chapter on pineal tumours, upon which the senior author has done so much work. Methods of classification of the glioma group are discussed, and the authors support the more conservative opinion of continuing the use of the terms "glioblastoma multiforme" and "malignant astrocytoma."

The authors have included one chapter by Professor C. E. Lumsden on tissue culture in relation to tumours, and this is excellent.

It seems a little hard for this reviewer to criticize, even though he has imbibed Greenfield's thoughts, having been a close colleague, but it would have helped pathologists in general if a chapter could have been added on pituitary tumours. These have been deliberately omitted, as is explained in the introduction.

Other minor comments that one can make are that it might have been easier to have arranged the order of Figs. 34 to 37 to correspond to the order of the description in the text of the various types of meningioma (not that they are numbered incorrectly); to maintain uniformity in the spelling of the word neurone; and to use the modern way for the spelling of such words as "criticize." Otherwise, no praise can be too high for this volume. It will have to be on every pathologist's bookshelf, and the authors have placed us all permanently in their debt, not only for their descriptive ability but also for the beauty of their illustrations.

J. N. CUMINGS.

Antibiotics in Medicine [*British Medical Bulletin*]. (Pp. 88; 4 art plates. 20s.) London: British Council. January, 1960.

An edition of the *British Medical Bulletin* devoted to antibiotics in medicine under the scientific editorship of Professor Garrod is a welcome event. With the large and ever-increasing number of antibiotics, which, as Professor Garrod points out in the introduction, are quite literally being unearthed from soil samples all over the world, the subject is inevitably confusing, and what makes "confusion worse confounded" is that each masquerades under a variety of trade names.

In this issue of the *British Medical Bulletin* some attempt is made at classification on two fronts, namely chemistry and antibacterial activity. The former is attempted in an admirably clear article by E. P. Abraham and G. C. F. Newton, and it is no reflection on their contribution to say that to the simple-minded clinical bacteriologist it is rather disappointing to see antibiotics with such widely divergent activity as cycloserine, penicillins, chloramphenicol, bacitracins, and polymyxins classified together.

Since antibiotics are derived from living organisms they inevitably display selective toxicity. This aspect is discussed by E. F. Gale, with special reference to the mode of action of penicillin, surface-active antibiotics, and chloramphenicol. The outstanding nature of Gale's own contribution to this subject is well known and this article needs no further commendation. In his conclusion Gale points out that studies of this nature have not so far helped very much in providing a rational approach to the design of chemotherapeutic agents. Nevertheless these studies have already done much to elucidate some of the mechanisms of bacterial metabolism, as well as the biochemistry of protein synthesis, so that their fundamental importance cannot be over-emphasized.

The next contribution, by M. R. Pollock, is concerned with the scientific basis of drug resistance and points out possible lines of prevention. The clinical problems connected with this subject are discussed generally by E. J. L. Lowbury, and from the point of view of the treatment of tuberculosis by John Crofton. All three contributions are worthy of close study.

The "Principles of Therapeutic Use" are described by L. P. Garrod and E. F. Scowen. As might be expected from the authors, the contribution is full of wisdom, perhaps not least in its opening sentence, which is as follows: "The first decision to be made when antibiotic treatment is contemplated is whether it is necessary at all." The rest of the article outlines the basis of "well-directed treatment limited to cases really requiring it" and includes a useful table of the comparative sensitivities of the common pathogens to the principal antibiotics.

Other contributions deal with the pharmacology of antibiotics, their preventive use in medicine and

surgery, combined therapy, the treatment of bacterial endocarditis, the dangers of antibiotic treatment, the laboratory control and uses of antibiotics, and finally the search for new ones. Space precludes individual mention of all of the contributions, but it is hoped that enough has been said to whet the reader's appetite for a most valuable collection of essays.

MARY BARBER.

Textbook of Microbiology, 17th ed. By William Burrows; with the collaboration of Richard Janvier Porter and James William Moulder. (Pp. xxiii+954; 301 figures. 98s.) Philadelphia and London: Saunders. 1959.

The 17th edition of Burrows' textbook maintains the high standard of its predecessors both in production and in the excellent survey it gives of every aspect of a rapidly advancing field. In many respects it can be compared only with two other American publications of the past two years, namely Zinsser's *Bacteriology*, 11th edition, 1957, and Dubos's *Bacterial and Mycotic Infections of Man*, 3rd edition, 1958. With the others it attempts, and very largely succeeds, in condensing into less than 1,000 pages all the most important work, beginning with cytology, thence through metabolism and immunity, to a study of the microbes themselves and their special role as disease agents. Especially useful are the clear and very readable accounts of bacterial metabolism, chemotherapeutic drugs and of variation and resistance, all of which have become increasingly important to bacteriologists.

Because of their size and the vast amount of detailed information given, books such as that under review must rank as reference works, indispensable to the laboratory worker but useful both to the teacher and student for the invaluable summaries which they contain. This textbook is too comprehensive for the medical student, unless he wishes to make microbiology his special study, and the same is true for clinical workers. One feels that in places the needs of the latter have been neglected, as in the following example. Staphylococcal cross-infection in hospitals is now a widespread and serious problem everywhere. The control of such infections and a study of their epidemiology by such measures as phage-typing are of immense importance to the clinical worker, yet the matter is hardly mentioned in the volume reviewed, a very serious omission which should be remedied in this and all other textbooks which hope to be of help and interest to the clinical pathologist.

(Late) E. S. DUTHIE.

Bacteriology for Students of Dental Surgery, 2nd ed. By R. B. Lucas and Ivor R. H. Kramer. (Pp. viii+288; 1 colour plate and 58 figures. 27s. 6d.) J. and A. Churchill. London. 1959.

This excellent little book contains all the information needed by the dental student on microbiology. The subject is approached from the medical rather than a narrow dental point of view, and there is an accurate, though necessarily brief, description of the major pathogens. One feels that the condemnation of the now discredited focal sepsis theory could be more outspoken, particularly as it is going to be read by future dental surgeons. The section on subacute endocarditis stresses the importance of dental hygiene in the evolution of this malady, but omits to suggest that antibiotic administration before and after dental treatment in patients with valvular disease might reduce the dangers of this serious condition. The book is nicely produced and illustrated, and the authors deserve praise for a fine piece of work.

S. D. ELEK.

Clinical Chemical Pathology, 2nd ed. By C. H. Gray. (Pp. viii+160; 28 figures. 14s.) London: Edward Arnold. 1959.

Professor Gray has revised and improved his popular little book on chemical pathology for its second edition. He has deleted many out-of-date methods of investigation (an example which could be followed by other textbook writers) and could still have deleted more. The U.M.I. of Heilmeyer and the Takata-Ara reaction have had their day, and the augmented histamine test should be standard practice. The information is sound, recent, and well presented, and the book is extremely lucid and readable, which would make it especially welcome for students. Pathologists will find it particularly useful in the selection of appropriate biochemical tests, and for support in advising clinical colleagues on the lack of value of rarely performed complex investigations. The guides to further reading can be commended.

The sections on diabetes and on liver function are particularly good; indeed, this contributes to the only real criticism of the book, namely a lack of balance. If Professor Gray had written other sections to the same scale as these, then he would have given us a textbook which could have been unreservedly recommended; but he would then have had to expand. Protein seems particularly neglected, and the electrophoretic patterns (p. 136) are not typical; where are the usually low albumin of myelomatosis, and the high α_2 globulin and low γ-globulin of the nephrotic syndrome? I also would not agree that analysis of serum potassium is of no value in investigating potassium depletion. Some of the terminology could be changed for the next edition, e.g., g. *not* gm.; proteinuria *not* albuminuria; *not* pint as equivalent to 500 ml.

Misprints are very few (I noticed 1 instead of 10 in the normal range of transaminases), and the book is cheap, well produced, and easy to handle. It should find a place in many a white-coat pocket.

D. N. BARON.

Protides of the Biological Fluids, 6th Colloquium 1958. Edited by H. Peeters. (Pp. ix+330; 171 figures, 60 tables. 45s.) London: D. Van Nostrand. 1959.

Every year since 1953 a conference has been held in Bruges and is concerned with proteins and electro-

phoresis. The first volume was small but has grown in size each year, so that now a book of some 300 pages is needed to cover the proceedings. Scientists from all over Europe pool their knowledge, and the meeting in 1958, the report of which is covered in this book, reveals how far the subject has grown. All chemical pathologists should certainly read it and will profit considerably.

The papers are recorded in one of three languages —French, German, and English—but there is a summary in English at the end of each communication. These reports are divided into those on general topics, on techniques, on macroglobulins, on various protein fractions associated with reproduction and on the digestive system and on proteins in disease. There are also reports on two round-table conferences held during the meeting. The standard of presentation is high, and it would be invidious to select a few as being of special merit, for so many are worthy of consideration. The detailed study of the papers will be of invaluable help to all those working on proteins and on electrophoresis. J. N. CUMINGS.

Chemical Quantitation of Epinephrine and Norepinephrine in Plasma. By William Muir Manger, Khalil G. Kakim, and Jesse L. Bollman. (Pp. xiv +398 ; 41 tables, 70 figures, 3 appendices. 87s. 6d.) Oxford: Blackwell Scientific Publications ; Illinois. Charles C. Thomas. 1959.

The amount of adrenaline and noradrenaline circulating in plasma is minute—of the order of a few micrograms per litre. As might be expected, chemical methods of assay are difficult, exacting, and tedious. Most pharmacologists tend to view them with some suspicion, for when it comes to the final criterion, the biological activity of the substance estimated, there are often marked discrepancies between results obtained by chemical and pharmacological assay.

The authors, who, to give them credit, are well aware of these limitations, have used one of the best of the chemical methods available, the fluorimetric technique of Weil-Malherbe and Bone, to carry out an exhaustive and painstaking study of plasma levels in a variety of clinical and experimental conditions.

Despite the unfortunate delay in publication, which has precluded mention of much of the latter-day break-through in the field of caechol amine metabolism, this is a useful book for the specialist.

M. SANDLER.

Experimental Allergic Encephalomyelitis and the "Auto-Allergic" Diseases. By Byron H. Waksman. (Pp. 87 ; 30 figures ; Sw. Fr. 15.) Basel and New York: S. Karger. 1959.

This monograph, published as a supplement to Volume 14 of the *International Archives of Allergy and Applied Immunology*, surveys in its first part the pathology of experimental allergic diseases. Experimental allergic encephalomyelitis, neuritis, auto-allergic diseases of the lens, uveitis, orchitis, thyroiditis, and iso-immune adrenalitis are described ; and clinical conditions with features resembling these experimental lesions, such as demyelinating diseases and post-vaccinal encephalomyelitis, mumps orchitis, Hashimoto's disease, and non-tuberculous granuloma of the suprarenal glands, are discussed.

The second part of the monograph is devoted to a discussion of the nature and pathogenesis of "auto-allergic" disease, that is, disease arising as a result of an immunological response to an individual's or species' own tissue. Immunologically speaking, the experimental conditions are all regarded as examples of delayed hypersensitivity, with the same fundamental lesion. Though the antigens are structurally very different (thyroglobulin, lens protein, proteolipid of myelin, and carbohydrate of sperm), Dr. Waksman considers that they have one major point of similarity, separation from the vascular bed and the connective tissue by more or less impermeable cellular barriers, such as the pial-glial membrane and the follicular thyroid epithelium, or by poor lymphatic drainage. This physiological remoteness, together with perhaps the special nature of their metabolism, prevents the antigens from reaching the blood stream in more than negligible concentrations, so that the immune apparatus treats them as foreign substances. If delayed reactivity results from this an "auto-allergic" disease process develops, in which a perivenular inflammatory reaction of mononuclear cells is the primary event, with a secondary parenchymal destruction. As the antigen persists, the disease tends to be prolonged or remittent.

Immunological evidence for the clinical parallels of most of the conditions discussed, with the possible exceptions of phako-anaphylactic endophthalmitis and Hashimoto's disease, is, as Dr. Waksman points out, far from conclusive. The still obscure mechanism of action of adjuvants deserves more discussion. The literature of the subject is surveyed fairly extensively, though remarks such as "but see 213a" and "the recent status of this disease is described in (196, 204, 208)" are not helpful. Otherwise this is a useful introduction to the literature of a difficult subject of increasing interest.

C. TREIP.

Coronary Heart Disease. By J. W. Gofman. (Pp. xx+353. 60s.) Oxford: Blackwell Scientific Publications ; Springfield, Illinois: Charles C. Thomas. 1959.

There are many books concerned with the coronary circulation. This is unlike any of the others, for it is exclusively about the aetiological theories of coronary artery disease. Not only from the clinical but also from the epidemiological outlook these theories are notoriously difficult to understand and correlate. Dr. Gofman very fairly quotes a large number of papers and tries to produce a reasoned approach to the prevention of coronary heart disease. He considers blood lipid factors, hypertension, the production of future clinical coronary disease, familial aspects, age, sex, obesity, diet, smoking, "hormones," stress, and possibilities of prevention.

He is meticulous in the differentiation between "atherosclerotic lesions" and "simple internal arteriosclerosis," and in the difference between morbid anatomical studies, biochemical blood analyses, and clinical findings. Unfortunately he analyses what is already known to describe a state of subclinical coronary heart disease, about which so little is known. However, to criticize informed speculation into such a vitally important problem is unfair. He provides all the data, and if the reader comes to a different conclusion it is for him to produce further work and change the argument.

The reviewer feels that this is an excellent, slightly "over-written" book, which is the work of an enthusiast and as such should be read by pathologists, although possibly not in its entirety. There are no illustrations.

RAYMOND DALEY.

Pancreatitis. By Herman T. Blumenthal and J. G. Probstein; Foreword by R. M. Zollinger. (Pp. xiv+379; 58 figures. 72s.) Springfield, Illinois: Charles C. Thomas; Oxford: Blackwell Scientific Publications. 1959.

This monograph presents a clinico-pathological correlation based on a study of 163 necropsies on patients dying from pancreatitis. It is divided into five parts dealing successively with statistical considerations, physiological disturbances, pathological anatomy, clinical features, and therapy. The section on pathology occupies some 75 pages and includes a full account of the normal embryology and anatomy of the pancreas. Clinical pathology is less fully dealt with, and it is perhaps unfortunate that very little indeed is said on the subject of chronic pancreatitis. Perhaps the greatest use of this book will be in the very full bibliography which it includes. This should render it invaluable as a source of references to anybody who is making a special study of pancreatic pathology, or indeed of any other aspect of pancreatitis.

T. CRAWFORD.

Correction

We regret that the review of "Polymyositis" published on page 92 of the January issue (*J. clin. Path.*, **13**, 92) was wrongly attributed to W. St. C. Symmers. It was in fact written by J. C. Sloper.

Association of Clinical Pathologists Broadsheets

The following broadsheets (new series) are published by the Association of Clinical Pathologists. They may be obtained from **Dr. F. Hampson, Department of Pathology, Royal Berkshire Hospital, Reading,** price 1s. each.

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1	The Disc Technique for the Rapid Determination of Bacterial Sensitivity to Antibiotics	1952	R. W. Fairbrother
2	Determination of Sensitivity of *M. tuberculosis* to Streptomycin	1952	R. L. Vollum
3	The Detection of Barbiturates in Blood, Cerebrospinal Fluid, Urine, and Stomach Contents	1953	L. C. Nickolls
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6	The Paul-Bunnell Test	1954	R. H. A. Swain
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22	Investigation of Haemolytic Anaemia	1959	J. G. Selwyn
23	The Dried Disc Technique for Bacterial Sensitivity Tests	1959	R. W. Fairbrother and J. C. Sherris

J. clin. Path. (1960), **13**, 187.

THE DIAGNOSIS, EPIDEMIOLOGY AND CONTROL OF URINARY INFECTION IN UROLOGY AND GYNAECOLOGY

BY

W. A. GILLESPIE, K. B. LINTON, A. MILLER, AND N. SLADE

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(RECEIVED FOR PUBLICATION JANUARY 1, 1960)

In a study of the urine after urological operations, quantitative bacteriological criteria for the diagnosis of infection were determined.

The bacteria responsible for urinary infection after urological or gynaecological procedures sometimes came from the same patient, but more often came from other patients by cross-infection. The principal final route of entry was the urethra or the indwelling catheter lying in it. These findings led to the elaboration of methods of destroying bacteria in the urethra before operation, improving the disinfection of cystoscopes, and preventing the entry of bacteria *via* the indwelling catheter. Application of these methods reduced the incidence of infection after prostatectomy from 83% to 6%, and after emergency bladder drainage for acute retention of urine from 73% to 10%. A smaller improvement followed the introduction of closed bladder drainage after gynaecological operations.

Urinary infection is a common complication of operations on the bladder and prostate and of drainage by indwelling catheters. This paper deals with the diagnosis and epidemiology of the infections and summarizes the results of preventive measures, some of which have been described more fully elsewhere (Miller, Gillespie, Linton, Slade, and Mitchell, 1958; Miller, Linton, Gillespie, Slade, and Mitchell (1960).

Methods of preventing infection were studied in patients who had sterile urine on admission to hospital and were not given prophylactic antibiotics or chemotherapy. There were three groups of patients:

(1) Men admitted under the care of members of one urological team with beds in both hospitals, for prostatectomy by the transurethral or the retropubic method. (Patients with suprapubic or perineal drainage of the bladder were excluded.)

(2) Men with acute retention of urine, treated as emergencies by indwelling catheter.

(3) Women who had gynaecological operations necessitating continuous bladder drainage by indwelling catheter.

The clinical effects of urinary infection were studied also in some patients whose urine was already infected before prostatectomy or who were admitted to wards where special measures to prevent infection were not yet in use.

Diagnosis

Urine was generally cultured within one hour and never more than two hours after collection, except for some of the initial samples which were cultured after overnight storage in the refrigerator.

Males.—The urine was examined daily (except on Sundays), starting from the day before operation, or from the first urine withdrawn from patients with acute retention. Urine was always obtained at operation by means of a cystoscope or catheter. Except for the first specimens from acute retention cases, all were collected by one of the investigators.

Many patients, when confined to bed, were unable to co-operate in the collection of "mid-stream" urine. For the sake of uniformity, therefore, specimens were passed direct into sterile jars, without prior cleansing of the glans penis and without discarding the first flow. When possible, large volumes were collected, usually 100 ml. or more, so as to minimize the effect of contamination from the urethra. Urine from patients on continuous drainage was withdrawn from the tube (after disinfecting it with alcoholic iodine) by means of a sterile syringe and needle.

Females.—Catheter specimens were obtained at operation. Thereafter, urine was taken each day from the drainage tube, as described above, and, after the indwelling catheter was removed, several "mid-stream" specimens were collected by a modification of the method of Hart and Magee (1957), in which the vulva was cleansed with sterile saline instead of

disinfectant. Also, catheter specimens were obtained when residual urine was drained from the bladder.

Laboratory Examination of Urine.—After mixing, 10 ml. was centrifuged, and wet and Gram-stained smears of deposit were examined. Standard loopfuls of uncentrifuged urine were inoculated on well-dried blood agar and MacConkey plates. The loop was kept vertical when withdrawn from the urine, and held approximately 1/150 ml. Six loopfuls were placed separately on each plate; five were allowed to soak into the medium without spreading and the sixth was spread over the rest of the plate. After aerobic incubation for 24 hours at 37° C., colonies were counted, if less than 50 per loopful. When the colonies were more numerous, that is, with counts of more than 7,500 per ml., the growth was recorded as "moderate" or "profuse."

The sensitivity of the bacteria to antibiotics and sulphonamide was determined by a disc method (Fairbrother, 1952) and by the method of Harper and Cawston (1945). Organisms were identified by appropriate cultural and biochemical methods. Fermentation tests with adonitol and inositol were used to distinguish Klebsiellae from Escherichiae (Talbot, Cunliffe, and Gower, 1957). Most strains of *Staphylococcus aureus* were phage-typed (Williams and Rippon, 1952), and many strains of *Escherichia coli* were typed by means of colicines (Linton, 1960).

Criteria of Infection.—Infection was diagnosed when two or more successive daily specimens of urine contained more than 3,000 bacteria per ml. Occassionally, a single specimen contained many bacteria, but subsequent specimens were normal. This did not constitute an infection; it sometimes happened soon after withdrawing the indwelling catheter from a prostatectomy patient whose urine had hitherto been sterile, the organisms apparently having multiplied locally in the clot and debris near the operation site.

The limit of 3,000 organisms per ml. was based on the results of culturing urine taken on the day before operation from 263 men whose urine taken by cystoscopy next day was found to be sterile. Of the pre-operative specimens, 138 gave no growth, 80 gave counts of between 1 and 750 colonies per ml., 39 yielded 750 to 3,000 colonies per ml. and in 21 of these the organism was *Staphylococcus albus*. Six specimens gave counts between 3,000 and 4,500 per ml., but in five the organism was *Staph. albus*.

The diagnostic limit of 3,000 organisms per ml. was slightly higher than that adopted by Sanford *et al.* (1956), who took 1,000 per ml. as the upper limit for normal urine. Their observations, however, were based on carefully collected "mid-stream" specimens, less subject to contamination than those used in the present investigation. On the other hand, Kass (1956) suggested the much higher limit of 100,000 organisms per ml.; but this was based on established infections. In the present investigation, an infection was considered to start when the responsible organisms first appeared in the urine, even if they were initially fewer than 3,000 per ml. Although the organisms were often scanty at the beginning of an infection, their numbers increased greatly during the next few days and almost invariably soon exceeded the limit of 100,000 per ml. suggested by Kass.

Hitherto, the diagnosis of infection in women has been based on the same criteria as in men, but it is hoped later to adopt standards based on a current investigation of catheter and "mid-stream" specimens from women.

Bacteriological Results

Before special steps were taken to reduce the incidence of infection, the organisms most commonly isolated from the urine of male patients were (in order of frequency) coliform bacilli, *Proteus*, *Staph. albus*, *Streptococcus faecalis*, *Pseudomonas pyocyanea*, and *Staph. aureus*. Many of the infections resulted from cross-infection; this was shown by the antibiotic resistance of the bacteria, by the results of typing *Esch. coli* and *Staph. aureus*, and by the occurrence of several "runs" of infection after prostatectomy caused by particular organisms (Gillespie, 1956; Linton, 1960).

Nearly all the infections in women were caused by coliform bacilli, *Proteus*, or *Str. faecalis*, and many of the organisms were resistant to several antibiotics. Mixed infections were common in both sexes.

Other workers have found the *Klebsiella* species to be responsible for much of the cross-infection by coliform bacilli in urological wards (Ørskov, 1952, 1954,; Dutton and Ralston, 1957). These organisms appear to develop resistance to antibiotics more readily than *Escherichiae* (Talbot *et al.*, 1957; Lattimer, Seneca, Zinsser, and Troc, 1959). The two groups of coliform bacilli isolated from infections in the Bristol Royal Infirmary showed a similar difference. Thus, of 41 *Klebsiellae*, 38 (93%) were resistant to one or more of the drugs streptomycin, tetracycline, chloramphenicol, and sulphonamide, compared with only 23 (29%) of the *Escherichiae*.

Most strains of *Staph. aureus* showed multiple antibiotic-resistance and were evidently similar to those responsible for wound infections in the same wards (Gillespie, Alder, Ayliffe, Bradbeer, and Wypkema, 1959).

Pyuria.—During the first two to three days after operation, the urine commonly contained a moderate number of pus cells, even in the absence of infection. At other times, sterile urine contained few pus cells, and the upper limit of normal for wet preparations was taken to be 15 cells per high-power field. More than this constituted pyuria.

In the early stages of an infection, there was often no pyuria. But within a few days pus cells nearly always became numerous. Of 281 patients with infected urine (including a few who were infected on admission), 269 developed pyuria at some stage. Of the 12 who did not, six were infected by *Staph. albus*, four by *Str. faecalis*, and two had chronic infections caused by small-colony coliform bacilli which probably were less virulent than normal strains (Gillespie, 1952).

The relationship between the infecting organisms and the pyuria was investigated in urine infected by single strains (Table I). *Staph. albus* and *Str. faecalis* were less often accompanied by pus, and

TABLE I
INCIDENCE OF PYURIA WITH DIFFERENT ORGANISMS

Organism	No. of Specimens of Urine Yielding Pure Cultures	Specimens Showing Pyuria
Staph. aureus	40	38 (95%)
Proteus sp.	47	43 (91%)
Coliform bacilli	99	87 (88%)
Other Gram-negative bacilli..	67	58 (87%)
Str. faecalis	22	16 (73%)
Staph. albus	80	54 (67·5%)

probably were less pathogenic than the other organisms. *Staph. albus* is often present as a commensal in the normal urethra (Shackman and Messent, 1954). Sometimes, however, it causes a definite though clinically mild infection, with numerous cocci and pus cells in the urine. But it was often difficult to decide whether *Staph. albus* was playing a pathogenic role, and therefore infections by this organism were not counted in the present investigation.

Incidence and Clinical Effects of Infection

The great majority of patients developed urinary infection within a few days of operation or insertion of an indwelling catheter (Table II). There was no appreciable difference between the incidence of infection after prostatectomy by the transurethral and the retropubic methods, but bacteria appeared to invade the blood stream more

TABLE II
INCIDENCE OF URINARY INFECTION DURING CONTROL PERIODS IN PATIENTS WITH URINE STERILE ON ADMISSION TO HOSPITAL

Treatment	No. of Patients	Patients Infected
Prostatectomy (transurethral and retropubic)	89	74 (83%)
Acute retention treated by indwelling catheter	26	19 (73%)
Gynaecological operations with indwelling catheters	41	40 (98%)

readily after the transurethral procedure (C. H. Talbot, personal communication). This difference was similar to that demonstrated by Grey (1955).

After prostatectomy, the infections usually lasted for at least three months and often for much longer; and, although as a rule not clinically severe, they usually caused pyrexia. An important minority of the patients suffered the serious, and sometimes fatal, consequences of acute pyelonephritis, septicaemia, and bacteriaemic shock. The removal of the indwelling catheter often precipitated a transient bacteriaemia (Miller *et al.*, 1958; Slade, 1958).

Several authors have emphasized the danger of invasion of the blood stream by bacteria from the infected urinary tract (Scott, 1929; Waisbren, 1951; Hall and Gold, 1955; Spittel, Martin, and Nichols, 1956; Ezzo and Knight, 1957). Their warnings have been borne out by experience in the surgical wards of these hospitals, where serious consequences of cross-infection, particularly septicaemia, have been caused more often by urological infection than by the staphylococcal infection of wounds which has received much attention in recent years. Such complications can be avoided only by preventing urinary infection in all urological patients.

Epidemiology

Measures to prevent cross-infection should be based, if possible, on epidemiological knowledge. In practice it is usually easy to suggest many sources of the organisms responsible for a particular outbreak, and many routes by which they may have spread; but it is often difficult to decide which are important. Obvious faults in ward and theatre techniques, and in the use of antibiotics, should be remedied. But in a particular situation it is of primary importance to identify the principal sources and routes of infection, and to concentrate on dealing with them. It is not necessarily enough to apply remedies which have been successful in other circumstances.

In the present investigation, many sources and routes of spread were found. The organisms mostly came from other patients; some came from the patient's own body (Gillespie, 1956; Linton, 1960). But there was one important feature common to all cases: the susceptible tissues, the bladder and prostatic bed, were situated deep in the body, and organisms generally could reach them only via the urethra. (This was different from staphylococcal cross-infection in general surgical and maternity units, where the susceptible tissues are mostly superficial and can be reached

by the staphylococci in many different ways.) It thus appeared that urological infection could best be prevented by excluding bacteria from the urethra and indwelling catheter before, during, and after operation. Methods of prevention were first tested in the prostatectomy patients, and the lessons learned from them were applied to the others.

Results of Precautions

Infection after Prostatectomy.—Because the causative organisms could often be isolated from ward furniture, blankets, etc., but were difficult to find in the theatre, the infections were at first thought to be post-operative, caused by organisms entering through the drainage tube and indwelling catheter which was left *in situ* for two or three days. Open drainage was in routine use. The lower end of the tube often became heavily contaminated from dipping intermittently into the urine which collected in an open vessel by the bedside. It was considered that bacteria were carried up the tube, against the flow of urine, by air bubbles which frequently ascended in it. That this could happen was confirmed experimentally. Moreover, when blood clot obstructed the catheter it was washed out by means of a sterile bladder syringe, a procedure which is difficult to perform aseptically. It was also thought that infection might be caused by bacteria which proliferated in the excess of secretion which often collects between the urethral mucosa and the outside of the catheter. This possibility was also suggested by Kass and Schneiderman (1957). To investigate it, swabs were taken from the lower end of the urethra, during drainage, and the organisms which they yielded on culture were compared with those which later caused urinary infection. The results were inconclusive (Gillespie, 1956). But the subsequent discovery that post-operative infection could usually be prevent by excluding organisms from the lumen of the catheter showed that the " extra-catheter " route was not important.

The first preventive measure was brought into use in the Bristol Royal Infirmary in November, 1955, by substituting closed drainage for open drainage. This made no difference to the incidence of infection. The apparatus was then modified to allow the bladder to be washed out without opening the system, but, despite this, more than half of the patients still became infected (Fig. 1). However, most of the infections now developed very soon after operation and were thought to originate in the theatre. Patients who escaped infection at operation usually remained uninfected afterwards, so long as the bladder was drained and irrigated by the closed aseptic method (Miller *et al.*, 1958).

The obvious next step was to review techniques in the operating theatre. It was found that the resectoscopes and cystoscopes, which could not be boiled, were being " disinfected " (after washing and taking them apart) by immersion in flat dishes of mercury oxycyanide solution, which is a poor disinfectant. The horizontal position of the instrument during this treatment may sometimes have prevented the fluid from completely filling the interior. A mixed aqueous solution of chlorhexidine (" hibitane ") 1/5,000 and mercury oxycyanide 1/2,000 was found to be a much better disinfectant than oxycyanide alone and slightly better than chlorhexidine alone (Miller *et al.*, 1958). The use of this solution, which was kept in tall cylinders in which the instruments could be immersed vertically, was followed by a sharp fall of the infection rate, to about 20% (Fig. 1).

Most of the infections that still occurred were caused by bacteria with normal antibiotic-sensitivity patterns, some of which resembled strains isolated from the urethra before operation and were probably pushed into the bladder at operation. The next step, therefore, was to attempt to disinfect the urethra before operation. As soon as the patient was anaesthetized, 5–10 ml. of 1/2,000 solution of chlorhexidine in glycerine was injected into the urethra and held there for about 10 minutes before operating. After this final precaution the incidence of infection fell again, to about 6% (Fig. 1).

Despite the greatly reduced incidence of infection following the introduction of the combined precautions, the value of closed drainage and irrigation was still in some doubt. The work was therefore repeated in Southmead Hospital, where the basic techniques were the same as those formerly in use in the Infirmary. Similar precautions were introduced, but in a different order. First, both of the " theatre precautions " were introduced simultaneously (the disinfection of the urethra and the disinfection of cystoscopes by means of chlorhexidine without oxycyanide). The resulting small improvement was similar to that which had followed the post-operative precautions at the other hospital. But the subsequent addition of closed drainage and irrigation brought about a marked improvement (Fig. 2), almost identical with that finally achieved in the Infirmary.

These results showed that each of the precautions was important, but that a substantial improvement could be achieved only by using

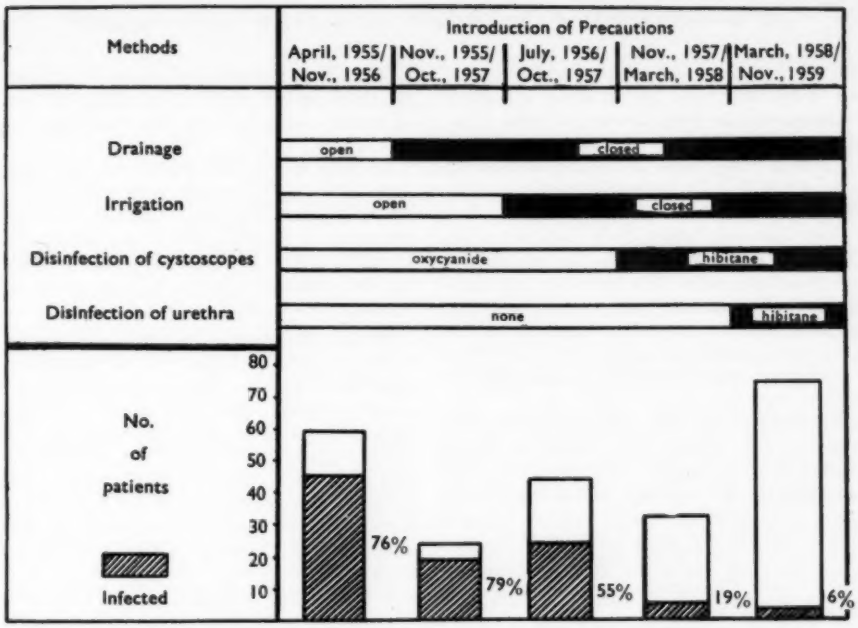

FIG. 1.—Effect of extra precautions on the incidence of urinary infection after prostatectomy in the Royal Infirmary.

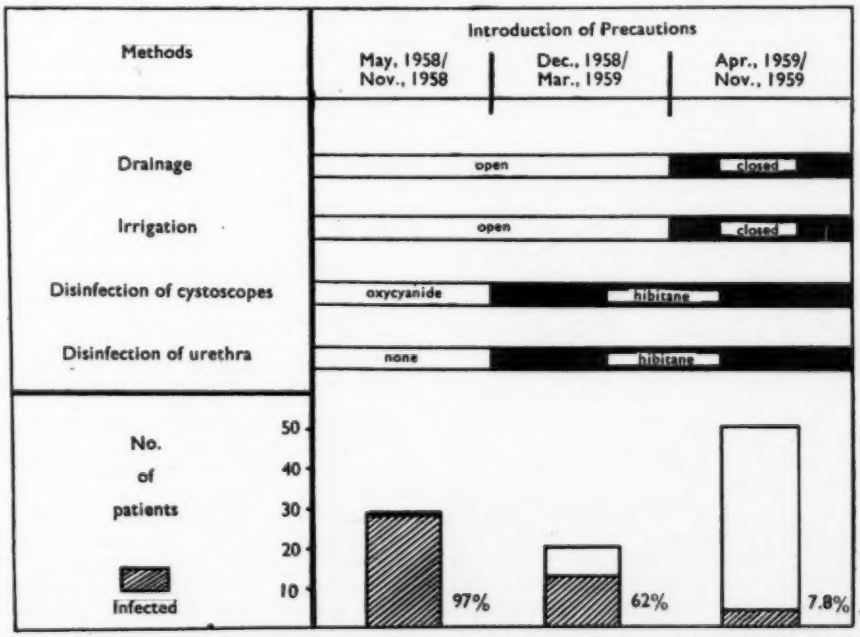

FIG. 2.—The effect of extra precautions on the incidence of urinary infection after prostatectomy in Southmead Hospital.

them in combination, when the incidence of infection in the two units fell to about 6% compared with more than 80% formerly (Table III).

In about 10% of the patients, the closed drainage system failed, usually because of profuse bleeding. Unless such patients were watched constantly, and frequently irrigated in the first hour or two after operation, their catheters often became so firmly blocked by clot that no method of irrigation was effective. The drainage apparatus then had to be dismantled, a new catheter inserted, and infection usually followed. These cases presented a surgical rather than a bacteriological problem, and showed the importance of haemostasis.

The overall infection rate among patients who were put on full precautions, including those whose closed drainage failed, was 12% (Table III). None developed septicaemia or bacteriaemic shock.

TABLE III
SUMMARY OF RESULTS WITH PROSTATECTOMY PATIENTS

Hospital	Infections/Total Patients	
	With No Additional Precautions	With Full Precautions
Infirmary	45/59 (76%)	4/75 (5·3%)
Southmead ..	29/30 (97%)	4/51 (7·8%)
		Results when closed drainage failed because of excessive bleeding 8/12 (67%)
Totals ..	74/89 (83%)	16/138 (11·6%)

The importance of blood-stream infection as a complication of urological procedures was brought out by an analysis of all the cases of bacteriaemia and septicaemia which were diagnosed by blood culture in the Bristol Royal Infirmary during the year ending October 31, 1959. Of the total of 33 cases, 12 (36%) were complications of operations on the urethra or bladder, or of the passage of sounds. None of these 12 patients had been treated with the full precautions described above; many already had urinary infections before they came to operation. The organisms isolated from their blood were coliform bacilli (four cases), *Proteus* sp. (three), *Staph. aureus* (two), *Ps. pyocyanea* (one), *Str. faecalis* (one), and *Staph. albus* (one). In every case, a similar organism was isolated from the urine. Four strains were abnormally resistant to antibiotics. Although many of the patients already had infected urine before operation, in at least 10 cases the organisms invading the blood stream were newcomers which had infected the urine since admission to hospital. It therefore appears important to avoid added infection in all patients admitted for urological operations, whether the urine is already infected or not.

Because of the wide variety of organisms which may cause blood-stream infection originating in the urinary tract, and because they may be resistant to several drugs, the initial treatment should include injections of two or more antibiotics, pending the identification of the causative strain.

Bladder Drainage for Acute Retention of Urine.—Many patients came to prostatectomy with established urinary infections which had usually started during previous catheter drainage or instrumentation, in these hospitals or elsewhere. Except for a few patients who were treated by immediate prostatectomy, the routine emergency treatment of acute retention was to drain the bladder by an open method, with an indwelling catheter and drainage tube which were freshly boiled before use.

A controlled trial was carried out in order to evaluate a simple method of closed drainage, the essential feature of which was that the end of the drainage tube was kept below the surface of formalin in a Winchester bottle (Miller *et al.*, 1960). This maintained sterility of the urine in the bottle, and was a fluid trap to exclude air bubbles. When the bottle became full, the tube was clamped while it was transferred to a new bottle. If the patient left his bedside, he carried his bottle with him. The results are summarized in Table IV. Patients with open drainage quickly became infected, whereas most of those with closed drainage did not.

TABLE IV
URINARY INFECTION DURING CONTINUOUS BLADDER DRAINAGE FOR ACUTE RETENTION

Method of Drainage	Infections/Total Patients
Open	19/26 (73%)
Closed	5/49 (10%)

Gynaecological Operations.—Some operations for vaginal repair and vaginal hysterectomy necessitated drainage of the bladder by indwelling catheter for a few days afterwards. An open drainage method was used. Subsequently, during the remainder of the post-operative period, a catheter was passed when necessary to remove residual urine. The frequency of catheterization varied greatly from patient to patient. In a preliminary investigation, it was found that nearly all the patients developed well-marked urinary

TABLE V

URINARY INFECTIONS AFTER GYNAECOLOGICAL OPERATIONS IN RELATION TO METHODS OF DRAINING THE BLADDER

Method	No. of Patients	Infected Within 18 Hours after Operation	"At Risk" for Infection Later	Infection Developed During		Total Infected
				Remainder of Catheter Drainage	Subsequently	
Open drainage	41	7	34	29* (85%)	4	40 (98%)
Closed ,, ...	25	7	18	5* (28%)	7	19 (76%)

* χ^2 (corrected) for the difference in the incidence of infection of patients on closed and open drainage during this period was 14·75; $P<0·001$.

infections (Table II), often accompanied by clinical disturbance and sometimes requiring chemotherapy. Some (not included in this series) were given prophylactic antibiotics, an undesirable practice which, however, sometimes prevented infection.

From March 25, 1959, a controlled trial was carried out to compare the incidence of infection with closed and open drainage. Alternate patients in one ward were given bladder drainage by one or other method. While the indwelling catheter was *in situ* there were far fewer infections with closed drainage, except during the immediate post-operative period when the incidence was high in both groups (Table V). Most of the early post-operative infections were probably caused by organisms introduced by the passage of catheters before or during operation. After the removal of the drainage tube, the incidence of infection was again similar in the two groups, probably because of the intermittent catheterization to which both were subjected. Contamination of the catheters was not a likely cause of infection, since they were freshly boiled before use. It seemed probable that both the post-drainage infections and the immediate post-operative infections were caused by organisms lying in the urethra which were pushed into the bladder by catheterization. This conclusion was supported by a comparison of the streptomycin sensitivity of the coliform and proteus bacilli causing infection at different periods after operation (Table VI). Most of the infections during open drainage were caused by streptomycin-resistant bacilli which probably came from the environment by cross-infection, whereas most of the other infections were caused by streptomycin-sensitive strains.

TABLE VI

STREPTOMYCIN RESISTANCE OF COLIFORM AND PROTEUS BACILLI CAUSING URINARY INFECTION AFTER GYNAECOLOGICAL OPERATIONS

	Streptomycin-resistant		Streptomycin-sensitive	
	Coliform Bacilli	Proteus	Coliform Bacilli	Proteus
Infections within 18 hr. of operation	0	0	6	3
Infections during drainage { Open	18	10	2	5
Infections during drainage { Closed	0	0	3	1
Subsequent infections ..	1	0	8	2

It is clear that closed drainage, though an important precaution, gave incomplete protection against infection. The passage of catheters after the drainage period should be kept to a minimum, and the procedure made as safe as possible when it has to be performed. Investigations are now in progress to determine whether the risk of infection can be reduced by instilling a disinfectant into the urethra before catheterization.

Discussion

There can be no doubt of the importance of avoiding urinary infection in all urological and gynaecological patients. In addition to the harm suffered by some of the patients, a high incidence of infection provides a temptation to employ a prophylactic antibiotic "cover" for operations; and some of the infections require treatment with antibiotics. A reduction in the incidence of infection, therefore, should diminish the consumption of antibacterial drugs in the wards and hence the spread of antibiotic-resistant strains.

In addition to the acute complications which may follow post-operative urinary infections, there is also the possibility that some patients may progress to chronic pyelonephritis. It is particularly important, therefore, to avoid infection in the comparatively young women who come for gynaecological operations.

Even the simple passage of a catheter may infect the bladder in either sex. Clinical pathologists, therefore, should be prepared to report on "mid-stream" specimens of urine from women as well as men and only to ask for catheter specimens in exceptional circumstances.

The routes by which organisms may reach the bladder while it is being drained by an indwelling catheter have already been mentioned. The success of the closed method in preventing infection during simple drainage of the male bladder showed that the principal route of

infection was the lumen of the catheter and drainage tube through which air bubbles readily ascend and carry up bacteria, unless steps are taken to prevent them. Infection in these patients can rarely if ever have been caused by organisms entering the bladder *via* the secretion which collects outside the catheter, as suggested by Kass and Schneiderman (1957). Kass and Sossen (1959) prevented infection by continuous irrigation with a weak solution of acetic acid, but this method would wash out bacteria from the bladder, no matter how they had entered it. However, the "extra-catheter" route may prove to be important in females and also in males whose bladders have to be drained for more than a week or so, which was the usual period of drainage after acute retention.

The proper disinfection of cystoscopes and resectoscopes was the simplest of all the precautions. Recent experience suggests that immersion for one or two minutes in a solution of chlorhexidine 0.5% in 70% alcohol (after thorough rinsing with water) may be as good as the slower method using aqueous chlorhexidine solution; but the margin of safety is small. The method of pasteurization described by Francis (1959) should be even better, and has the added advantage of killing tubercle bacilli.

The adoption of closed drainage had an important secondary effect in reducing the reservoirs of antibiotic-resistant bacteria which had often caused cross-infection. Formerly, bacteria had multiplied freely in the open vessels into which the urine was drained. Antibiotic-resistant strains, chiefly *Klebsiellae*, could frequently be found there in large numbers and must often have contaminated the hands of the nurses who emptied the vessels.

The risk of infection during catheter drainage is increased if the catheter has to be changed. Rubber catheters often have to be changed after a week or so, because of non-bacterial inflammation of the urethra. Bladder drainage for long periods should therefore be affected by means of a non-irritating catheter such as the narrow-bore plastic catheter described by Gibbon (1958).

We are grateful to the Board of Governors of the United Bristol Hospitals for a grant which enabled us to do this work, to Professor G. G. Lennon for his encouragement, to the clinicians who allowed us to study their patients, and to the nursing staffs for their assistance. Our thanks are also due to Dr. F. J. W. Lewis and Dr. S. T. Crowther for their help, and to Mr. L. J. Waller for much support in the laboratory work.

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TYRAMINE-PRODUCING STREPTOCOCCI IN NEONATAL DIARRHOEA

BY

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(RECEIVED FOR PUBLICATION NOVEMBER 11, 1959)

An outbreak of neonatal diarrhoea associated with large numbers of *Streptococcus faecalis* var. *zymogenes* is described.

A follow-up survey showed that 23.3% of 347 healthy babies carried this organism without ill effect.

The tyrosine decarboxylase activity of the streptococci from both sick and healthy babies was identically high.

It is suggested that the tyramine-producing activity of these streptococci was not the primary cause of the diarrhoea.

Tyramine is similar to histamine and possesses powerful physiological actions. It is produced from the amino-acid tyrosine by decarboxylation. The enzyme capable of doing this is called tyrosine decarboxylase (Hawk, Oser, and Summerson, 1947).

Gale (1940) demonstrated that certain strains of *Streptococcus faecalis* could produce tyramine, and he later (Gale, 1944) described how he had found large numbers of β-haemolytic, group D streptococci in the stools of infants involved in some outbreaks of neonatal diarrhoea. These streptococci had an unusually high tyrosine decarboxylase activity and when fed to infant rats resulted in a fatal diarrhoea. This syndrome could be imitated by feeding tyramine itself. Older rats were not susceptible to the action of either the organisms or the amine. He explained this by showing that tyramine oxidase was significantly deficient in infant tissue at birth but fully developed at the end of the third month.

Sharpe (1948) investigated 340 strains of streptococci from infants' faeces and estimated their tyrosine decarboxylase activities. She found that the *Strep. faecalis* varieties—*faecalis, liquefaciens,* and *zymogenes*—had a high carboxylase activity, whereas *Strep. durans* and *Strep. bovis* showed little activity.

Sharpe and Shattock (1952) typed serologically group D streptococci associated with outbreaks of neonatal diarrhoea. They listed 24 types and three subtypes (five types of *Strep. faecalis* var. *zymogenes*).

Sharpe (1952) examined the streptococcal flora of faeces in eight outbreaks of neonatal diarrhoea associated with non-haemolytic group D streptococci. She suspected *Escherichia coli* were involved in some of the outbreaks.

An Outbreak of Neonatal Diarrhoea at Oldchurch Hospital

Fifteen newborn babies in one of three nursery wards were found to be suffering from diarrhoea with loose green stools. They showed some loss of weight but no obvious dehydration. Two of these 15 babies died during the investigation.

Stool cultures showed a marked predominance of group D β-haemolytic streptococci with scanty growth only of *E. coli, Proteus* spp., and non-haemolytic streptococci. Some of the cultures showed aerobic flora composed entirely of β-haemolytic streptococci. Table I shows the results of daily faecal cultures on 14 babies. The scanty growths of *E. coli* isolated were tested with *E. coli* O.55, O.26, and O.111 antisera with negative results.

After 10 days the remaining babies were discharged. The ward was closed for a week and thoroughly cleaned.

Necropsy Findings.—One baby died at the onset of the investigation. The cause of death was acute pulmonary infection. *Strep. faecalis*

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TABLE I

GROWTH OF β-HAEMOLYTIC STREPTOCOCCI IN FAECAL CULTURES IN M.A. NURSERY

	Date							
	Mar. 4	Mar. 5	Mar. 6	Mar. 7	Mar. 8	Mar. 9	Mar. 10	
Baby 1 ..	++	+++	+++	++	+	+	+	Tc.
,, 2 ..	+++	+++	+++	+	+	+	++	
,, 3 ..	++	+++	+++	++	+	±	++	,,
,, 4 ..	+++	+++	+++	+++	±	0	0	,,
,, 5 ..	+++	+++	+++	+++	+ Died		—	
,, 6 ..	++	++	+++	++	++	+++	++	
,, 7 ..	++	+++	+++	+	0	0	++	,,
,, 8 ..	+++	Discharged		—	—	—	—	
,, 9 ..	++	+++	+++	++	+ Discharged		—	
,, 10 ..	+++	+++	++	0	0	±	++	,,
,, 11 ..	+++	+++	+++	+++ Discharged		—	—	
,, 12 ..	+++	+++	0	0	0 Discharged		—	
,, 13 ..	+++	+++	+++	++	+++ ,,		—	
,, 14 ..	—	+++	+++	0	—	±	0	,,

Tc.=treated with chloramphenicol from Mar. 7. +=degree of growth of streptococci in the faecal culture, i.e., ±=1 or 2 colonies. +=scanty growth. ++=moderate growth. +++=profuse growth (60–100% total flora). 0=no growth of β-haemolytic streptococci. —=no swab received.

TABLE II

BABIES IN NURSERY A.2 CARRYING β-HAEMOLYTIC STREPTOCOCCI IN FAECAL FLORA

	Date											
	Mar. 26	Mar. 27	Mar. 28	Mar. 29	Apr. 3	Apr. 4	Apr. 5	Apr. 6	Apr. 7	Apr. 9	Apr. 10	Apr. 11	Apr. 12
Baby A ..	0	0	0	0	—	—	—	—	—	—	—	—	—
,, B ..	0	0	+	0	++	++	0	—	—	—	—	—	—
,, C ..	0	0	0	0	—	—	—	—	—	—	—	—	—
,, D ..	+++	+++	+++	+++	—	—	—	—	—	—	—	—	—
,, E ..	—	—	0	0	+	+	++	—	—	—	—	—	—
,, F ..	—	—	+++	+++	+	++	++	—	—	—	—	—	—
,, G ..	—	—	—	0	—	0	0	0	—	—	—	—	—
,, H ..	—	—	—	++	+	0	++	++	++	++	—	—	—
,, I ..	—	—	—	++	+	+	0	++	—	—	—	—	—
,, J ..	—	—	—	0	0	0	0	0	0	0	—	—	—
,, K ..	—	—	—	0	0	0	0	0	±	++	+	—	—
,, L ..	—	—	—	+	0	0	0	0	—	—	—	—	—
,, M ..	—	—	—	—	+	±	+	0	0	++	+	++	++
,, N ..	—	—	—	—	++	++	++	++	++	++	++	—	—
,, O ..	—	—	—	—	0	0	0	0	0	0	0	—	—
,, P ..	—	—	—	—	0	++	+	0	0	0	++	++	+
,, Q ..	—	—	—	—	0	0	0	0	0	0	—	—	—

+=degree of growth of streptococci in the faecal culture, i.e., ±=1 or 2 colonies. +=scanty growth. ++=moderate growth. +++=profuse growth (60–100% total flora). 0=no growth of β-haemolytic streptococci. —=no swab received.

var. *zymogenes* was grown from the bronchus. The small gut appeared pink and inflamed, but no β-haemolytic streptococci were grown from it.

The second baby died six days later. The cause of death was multiple focal necroses of the liver, probably secondary to umbilical sepsis.

Epidemiological Investigation.—In the remaining two nursery wards rectal swabs from the babies were examined daily for evidence of β-haemolytic streptococci.

A sample of the results obtained from one nursery is shown in Table II.

Later it was decided to swab the babies from all three nurseries once only, on discharge. By the end of four months, 347 babies had been investigated. None of these babies showed evidence of enteritis (Table III).

TABLE III

RESULT OF INVESTIGATION OF FAECAL FLORA IN 347 HEALTHY BABIES

Ward	No. of Babies	No. of Babies with Group D Haemolytic Streptococci	No. of Babies with Profuse Growth of Group D Haemolytic Streptococci
M.A.	132	27 (20·5%)	16
A.1	104	24 (23·1%)	7
A.2	111	30 (27·0%)	12
Total	347	81 (23·3%)	32 (9·2%)

No further occurrence of diarrhoea was reported in the discharged group of affected babies. The investigation was closed after five months.

Bacteriology.—The haemolytic streptococci isolated from the 15 babies involved in the outbreak of diarrhoea were confirmed as *Strep.*

faecalis var. *zymogenes* by the Streptococcal Reference Laboratory, Colindale.

The 81 strains of β-haemolytic streptococci isolated from the healthy babies were examined for their biochemical reactions in parallel with the 15 strains of streptococci isolated from the affected babies.

All 96 strains of streptococci were identified serologically as group D. All showed growth at *p*H 9.6 and grew in broth containing 6.5% NaCl. All strains fermented mannite and sorbitol and failed to ferment arabinose and raffinose in serum-water media.

All the strains were heat resistant when tested for 30 minutes at 60° C. and they all grew on MacConkey agar.

The strains were all resistant to tellurite when grown on blood agar containing a final concentration of 1/2,500 potassium tellurite.

All 96 strains showed variable results in the liquefaction of gelatin and the fermentation of glycerol.

The 15 strains isolated from the affected group of babies showed one antibiotic sensitivity pattern, namely, sensitive to chloramphenicol and erythromycin, insensitive to penicillin, streptomycin, and the tetracyclines.

Of the remaining 81 strains, isolated from healthy babies, 25 strains showed this same pattern and 56 strains showed varying antibiotic sensitivity patterns.

The tyrosine decarboxylase activity of the streptococci was estimated by the method of Gale, as described by Sharpe (1948). Sharpe found that minute variations in the medium caused considerable *p*H differences and she tested each batch of medium with a reference strain of *Strep. faecalis* obtained from Gale, which under test conditions gave a difference of 1.5–1.8 *p*H units.

A strain of *Streptococcus faecalis* from the National Collection of Industrial Bacteria (N.C.I.B. 6783), which had been deposited by Gale as a reference strain for the estimation of tyrosine decarboxylase, was examined. In our media, this strain, under test conditions, gave a difference of 1.47 *p*H units.

All 96 strains of haemolytic streptococci isolated showed a high tyrosine decarboxylase activity.

The streptococci isolated from the 15 affected babies showed activities varying from 1.78 to 2.31 *p*H units. The streptococci isolated from the 81 healthy babies showed activities varying from 1.78 to 2.42 *p*H units.

Serological typing of the group D streptococci was not carried out.

Discussion

This outbreak of neonatal diarrhoea closely paralleled the outbreaks described by Gale (1944) inasmuch as a group of babies with diarrhoea was found to be excreting large numbers of β-haemolytic group D streptococci.

Gale's hypothesis that the diarrhoea of the babies was caused by the tyramine produced by the predominantly streptococcal faecal flora seemed very probable, and the probability seemed to be stronger when it was discovered that the streptococci, in this outbreak, had a high tyrosine decarboxylase activity.

But further examination of the faecal flora in all the babies born in this hospital over a period of four months showed that large numbers of these babies carried *Strep. faecalis* var. *zymogenes* in their faeces without ill effect. The tyrosine decarboxylase activity was as high in these streptococci as it was in the streptococci isolated from the infected babies. The varying antibiotic sensitivity patterns of these organisms suggested that more than one serological type of streptococci was carried.

The record of daily faecal cultures on one group of babies (extracted in Table II) showed that this organism usually established itself in the first or second day of the infant's life. The babies who had escaped infection by then usually remained free from the organism for the remainder of their stay in hospital (10 days).

None of the outbreaks of neonatal diarrhoea investigated by Sharpe (1952) had β-haemolytic streptococci as the predominant faecal flora. Only one outbreak investigated showed a marked prevalence of non-haemolytic group D streptococci.

Sharpe was not able to come to any definite conclusion and suggested: (1) That diarrhoeal conditions may have favoured the establishment of this type of streptococcus (tyramine producer) already present in small numbers in the intestine. (2) Unfavourable factors in managing infants may have rendered the gut particularly susceptible to this organism and their increased numbers may have caused the diarrhoea, possibly by means of the increased amount of tyramine produced by the almost pure streptococcal flora.

From the findings in our investigation it would appear that the carriage in the faecal flora of large numbers of highly active, tyramine-producing streptococci does not necessarily lead to diarrhoea and that some other factor or factors must be involved. Ross (1951) found that the tyrosine content of the large bowel in newborn babies is negligible.

It is possible that in this outbreak of diarrhoea the babies could have been accidentally fed large quantities of the organism by mouth, as Gale (1944) fed his young rats. Sharpe also considered this possibility and suspected the glucose-water which she could not check. We tested the glucose, teats, and bottles used, but found no streptococci present. Mothers of the babies and hospital staff associated with these babies were examined, but showed no evidence of group D β-haemolytic streptococcal infection.

The deaths of the two babies in the outbreak were undoubtedly due to systemic infection by the predominant organism in their heavily infected environment. The post-mortem appearances were characteristic of a systemic invasion by an organism of low virulence.

Grateful thanks are expressed to the Director of the laboratory, Dr. E. A. Atkinson, for his encouragement and for his necropsy reports which he made freely available.

We are also grateful to the Curator, National Collection of Industrial Bacteria, for helpful advice and for the reference strain of streptococci.

Without the hard work and co-operation of the staff of the Maternity Unit this work could not have gone forward.

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THE INFLUENCE OF MECHANICAL FACTORS ON THE STRUCTURE OF THE PERIPHERAL ARTERIES AND THE LOCALIZATION OF ATHEROSCLEROSIS

BY

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In a study of the peripheral arteries a period of development lasting until the end of the second decade of life is described. During this time there is evidence of medial hypertrophy and a continued formation of the longitudinal muscular cushions described in the foetus. Bands of longitudinal muscle also appear in the media of certain vessels. It is considered that the findings confirm the functional significance of foetal cushions and further suggest their relationship to the stresses of pulsation. In the later decades the tendency of atherosclerotic plaques to develop at the site of cushions is believed to reflect the importance of mechanical factors in the localization of this disease.

The finding of cushion-like thickenings composed of longitudinal muscle and elastic tissue in the walls of the popliteal and lower end of the brachial artery of the foetus was reported in a previous communication (Robertson, 1960). These occurred mainly at the mouths of branches and were found to be similar to intimal thickenings previously described in the coronary arteries of the foetus (Dock, 1946; Minkowski, 1947). It was believed that they represented the physiological reaction of the vessel wall to a pulsatile longitudinal stress, and their presence in only certain of the muscular arteries was attributed to the unrestricted movements of these vessels during pulsation.

Mechanical stresses have been considered by many to be important in the genesis of atherosclerosis, and those due to pulsation have been most often incriminated (Duguid, 1926; Moschcowitz, 1942). For this reason, it was thought that it would be of interest to study the subsequent development of foetal cushions in the peripheral arteries of the adult and to determine their relationship to atherosclerosis. During the course of this work it was observed that many of the changes which occur focally in the arteries of the foetus are continued in a more exaggerated form into early adult life. It is believed that this reflects a continuous process of arterial development which is the physiological response of the vessel to certain well-defined haemodynamic stresses.

Materials

The peripheral arteries of 81 subjects coming to necropsy were examined, their ages ranging from 1 month to 95 years. All those with clinical or post-mortem evidence of hypertension were excluded from the series.

A small segment of each artery studied was taken from the same site in each case. In the upper limb segments were taken from the brachial artery both at the level of the insertion of the deltoid muscle and in the antecubital fossa. In 65 cases, the radial artery at the wrist was examined. In the leg, segments were taken from the femoral artery in the upper part of the subsartorial canal, from the popliteal artery in the popliteal fossa, and from the posterior tibial artery just above the medial malleolus. Sections were stained with haematoxylin and eosin and with Weigert's elastic tissue stain and van Gieson.

The age distribution of the 81 cases is shown in Table I.

TABLE I

AGE DISTRIBUTION

Years	No.
0–10	16
11–20	8
21–30	8
31–40	7
41–50	7
51–60	9
61–70	7
71–80	8
81–90	8
91–100	3

Findings

During the first two decades of life three changes were seen to occur in the structure of the peripheral vessels. The formation of muscular cushions at branch orifices was continued from foetal life and extensive bands of longitudinal muscle developed in the media of the popliteal and lower brachial arteries. In the intima, the internal elastic lamina split into two lamellae to form the musculo-elastic layer.

In the first decade the muscular cushions previously described in the popliteal and lower end of the brachial artery of the foetus showed some increase in size but no other alteration in their appearance. By the end of the second decade, however, cushions had often been formed at the mouths of branches in the other peripheral vessels. They were especially prominent in the femoral artery. Here they frequently rivalled the popliteal cushions in size and were of the same structure, being composed of longitudinal muscle supported by a prominent mesh of elastic tissue (Fig. 1). In the posterior tibial and radial vessels, on the other hand, cushions were much less frequently found and often consisted of only small and inconspicuous nodes of longitudinal muscle. Simultaneously with the development of cushions throughout the peripheral vascular system the media of the popliteal and lower brachial arteries began to acquire bands of longitudinal muscle (Fig. 2). This change was not found in any of the other peripheral vessels and was much more marked and constantly present in the popliteal than in the brachial artery. These bands were at first quite narrow, but often extended more than half-way around the circumference of the vessel. During the second decade they became much broader and in some vessels their prominence was quite remarkable, longitudinal muscle bundles forming almost the whole width of the media (Fig. 3).

FIG. 1.—Femoral artery. Female, 19 years. Cushions of longitudinal muscle are present on either side of a branch. Weigert ×100.

Towards the end of the first and in the second decade the internal elastica began to split to form the musculo-elastic layer of intima. This occurred fairly uniformly around the circumference of the larger vessels. In the popliteal artery, however, splitting tended to occur first over bands of medial longitudinal muscle and the musculo-elastic layer was often thickest in these regions (Figs. 2 and 3). The manner in which the musculo-elastic layer was formed was quite distinctive. Initially, the internal elastica thickened and two component parts of it could be recognized with Weigert's elastic tissue stain. These two lamellae then began to move apart and even in the earliest stages of separation densely staining cell nuclei could be distinguished between them in many places (Fig. 4). As further separation occurred these nuclei could be identified as belonging to smooth muscle cells which were orientated mainly in the longitudinal axis of the vessel. Muscle cells could also be seen to be passing from the media into the newly formed musculo-elastic layer through fenestrae in the internal elastica in a manner similar to that described in the foetus.

At the end of the second and in the later decades the musculo-elastic layer of the intima presented a very varied appearance. In some cases a well-defined layer was present, but in others reduplication of the internal elastica had apparently not yet occurred or was only in its very early stages, even in vessels of the third or fourth decade. The appearances in some sections suggested an explanation for the seemingly tardy formation of this layer. In these, the elastica was duplicated around most of the circumference of the vessel, but in places large gaps had appeared in the outer elastic lamella so that here the musculo-elastic layer had become continuous with

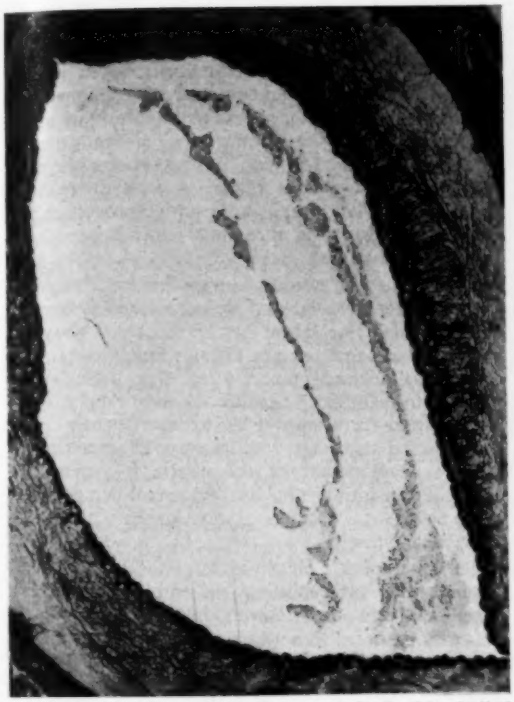

FIG. 2.—Popliteal artery. Male, 11 years. A band of longitudinal muscle in the media extends around much of the circumference of the vessel. A broad musculo-elastic layer covers it. Weigert ×65.

FIG. 3.—Popliteal artery. Female, 19 years. Longitudinal muscle bundles are prominent in the media. The internal elastica has split to form the musculo-elastic layer. Weigert ×150.

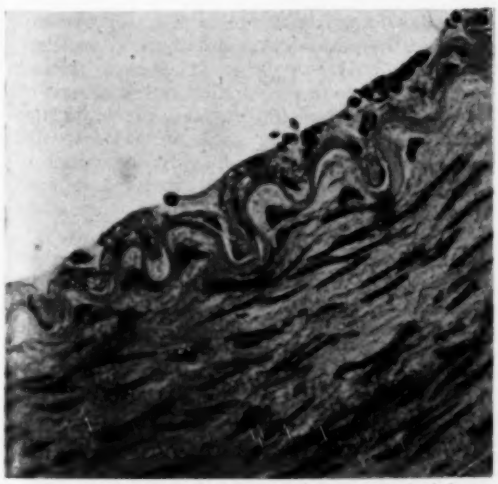

FIG. 4.—An early stage in the formation of the musculo-elastic layer. The internal elastica has begun to split and cell nuclei can be distinguished between its two components. Haematoxylin and eosin ×400.

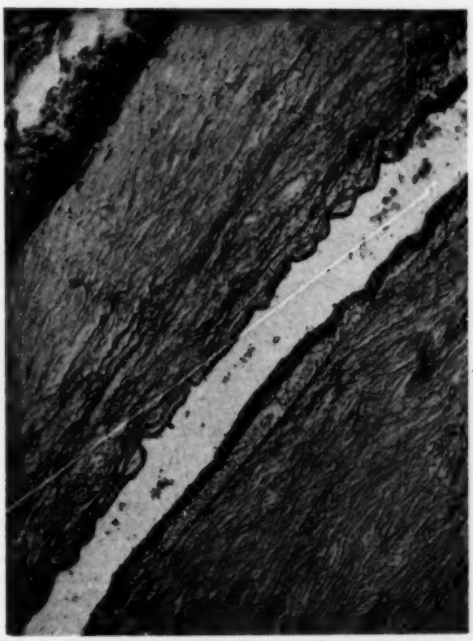

FIG. 5.—Femoral artery. Male, 33 years. Large gaps are present in the outer elastic lamella. In places the elastic stippling of the musculo-elastic layer is quite distinct, but in others the media has extended up to the lining elastic membrane. Weigert ×80.

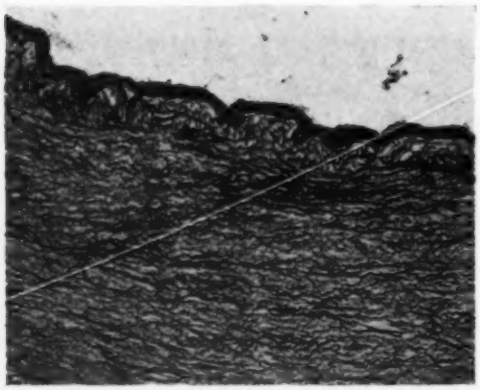

FIG. 6.—The elastic pattern of a former musculo-elastic layer can still be distinguished. Renewed splitting of the internal elastica is occurring. Weigert ×220.

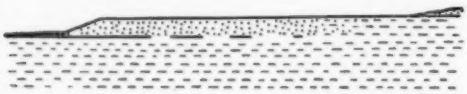

FIG. 7.—From left to right is illustrated the proposed sequence of the formation and incorporation of the musculo-elastic layer into the media. Circular muscle is represented by dashes, longitudinal muscle by dots, and elastic tissue by the continuous lines.

FIG. 8.—Brachial artery. Male, 35 years. Longitudinal muscular cushions are present on either side of a branch. The elastic hyperplastic layer has become thickest over them and already small plaques have been formed. Weigert ×70.

the media. In these places the intimal layer could often still be distinguished by virtue of the pattern of its muscle and elastic fibrils, but in parts it had lost its identity and merged with the media (Fig. 5). In other places renewed splitting of the internal elastica was sometimes evident over remnants of a former musculo-elastic layer (Fig. 6). These appearances suggest that the formation of this layer is a repetitive process and that each layer is in turn incorporated into the media as illustrated in Fig. 7.

During the first two decades the intimal changes in the posterior tibial and radial arteries differed from those in the larger trunks described above. Although in some cases a distinct musculo-elastic layer was formed, more often this layer was difficult to distinguish because the internal elastica had split to form several elastic membranes.

The third and later decades were characterized by the development of the elastic hyperplastic layer of the intima and by regressive changes in the muscular cushions and media of the peripheral vessels.

The hyperplastic elastic layer of the intima was formed by the splitting of multiple elastic lamellae from the internal elastica. As the process continued these elastic membranes became progressively finer until there was formed an elastic mesh in the interstices of which there were both smooth muscle cells and connective tissue. During the later decades the elastic hyperplastic layer became thicker and in places nodular. The nodular parts most often occurred over muscular cushions at the mouths of branches and over bands of medial longitudinal muscle (Fig. 8). Accordingly, in the posterior tibial and radial vessels it formed a fairly even layer and became thickest and very uneven in the popliteal artery because of the prominence of longitudinal muscle in this vessel. As the nodular areas increased in size they became vascularized, much more fibrous in character, and lipoid deposits appeared in their substance, completing their transition into atheromatous plaques.

During this period there was a progressive fibrosis of the media with atrophy of its longitudinal muscle. These regressive changes were only gradual in onset and did not become prominent until the fifth decade. Medial fibrosis tended to be most marked beneath developing intimal plaques at the mouths of branches. Here, to a large extent, it replaced the longitudinal muscle of the media so that eventually only remnants of the former muscular cushion could be distinguished beneath the plaque.

Discussion

Cushion-like thickenings composed of longitudinal muscle and elastic tissue occur in the walls of the popliteal, lower brachial, and coronary arteries of the foetus. They are found especially at the mouths of branches, and it was considered that their function was to buffer the stresses produced by the anchoring effect of the branch on the systolic elongation of the parent vessel. Their presence in only certain of the muscular arteries was thought to reflect marked pulsation of these vessels due to the lack of support they receive from surrounding tissues (Robertson, 1960).

It is believed that the changes which occur in the peripheral vessels during the first two decades of life emphasize the functional significance of the muscular cushions of the foetus. During this period it appears that the same remodelling process which was begun in the foetus is continued and becomes exaggerated. Muscular cushions continue to be formed and appear in even the small peripheral vessels. Extensive longitudinal muscle bands are developed in the media of the popliteal and, to a lesser extent, the lower brachial arteries. Gross, Epstein, and Kugel (1934) have described how longitudinal muscle also becomes prominent in the media of the coronary arteries by the second decade. The degree of development of these changes can be correlated with the severity of pulsatile stress. In the smaller arteries, where the pulse wave is flattened and less steep (Sodeman, 1956), muscular cushions are small. In those vessels, in which it has been suggested that pulsation is marked, cushions develop earlier, are much larger, and are reinforced by extensive longitudinal muscle bands in the media.

During the first two decades the musculo-elastic layer of the intima is also formed. There have been many interpretations of the significance of this layer. Some have considered it to be a manifestation of growth; others believe it to be degenerative in nature and an inevitable change due to age. MacLean (1929) was of the opinion that any splitting of the internal elastica was pathological as sometimes the coronary arteries of quite elderly subjects showed little evidence of it. Aschoff (1924) regarded the musculo-elastic layer as evidence of hypertrophy in response to a gradual rise in blood pressure. That such a rise does occur with age among the general population has been shown by Master, Dublin, and Marks (1950) and by others.

The results of this study support this last view. It has been described how the formation of this layer is a repetitive process which is primarily medial in origin and results in an increase in the thickness of this coat. Its absence in foetal life argues against it being a manifestation of growth, while its accentuation over medial longitudinal muscle bands tends to associate it with hypertrophy. Wolkoff (1924) has found that in rabbits, cats, dogs, and monkeys the musculo-elastic layer develops only at the margins of branches where also longitudinal hypertrophy is so prominent in the human subject. The manner in which the musculo-elastic layer is formed is also reminiscent of the elastic tissue changes which occur over developing foetal cushions. In both cases smooth muscle cells appear to split the internal elastica mechanically into lamellae which then become increasingly separated by a further ingrowth of medial muscle. In the foetus the outer lamella eventually becomes greatly interrupted and difficult to distinguish, while in the adult it disappears with a resultant increase in the width of the media. It is believed, therefore, that the development and subsequent incorporation of the musculo-elastic layer into the media reflect a hypertrophy of the circular muscle of the vessel in the same way as the foetal cushion represents a local longitudinal hypertrophy.

The repeated formation of the musculo-elastic layer could be expected to have a further result. Not only will it increase the width of the media but there will also be a reduction in the radius of the lumen of the vessel. According to Burton (1951), the law of Laplace applies to blood vessels. This states that the tension developed in the wall (T) is proportional not only to the excess of hydrostatic pressure in the lumen over the local surrounding tissue pressure (P) but also to the radius of the lumen (R), that is, $T = P \times R$. Thus, the repeated formation of the musculo-elastic layer constitutes a physiologically efficient method of compensating for a rise in blood pressure. Not only is the muscular bulk of the wall increased but also the radius of the lumen is decreased, so tending to reduce the tension in the wall and the need for hypertrophy.

The prominent part played by medial smooth muscle in the formation of the musculo-elastic layer has been described. This supports the observations of previous authors. Jores (1903, quoted by McMeans, 1915) believed that splitting of elastic tissue is accomplished mechanically by smooth muscle. Gross *et al.* (1934) and Altschul (1950) have both described the passage of smooth muscle from the media into the intima. Altschul described it in both human and experimental arteriosclerosis and considered that vascular

smooth muscle may possibly be capable of amoeboid movements. More recently, Morgan (1956) has confirmed that many of the smooth muscle cells of atherosclerotic plaques are derived from ingrowths of medial muscle.

During the first two decades the musculo-elastic layer of the posterior tibial and radial arteries was difficult to distinguish because of the frequent formation of several elastic lamellae between which were many smooth muscle cells. It seems possible that this change represents an exaggerated form of the musculo-elastic layer. Mitchell (1956) has observed that the smaller peripheral arteries have a more profuse vascular innervation and relatively thicker muscular coats than the more proximal trunks. If active vasomotor function requires these vessels to have an unusually thick muscular coat it could be expected that a rise in blood pressure would result in a more urgent stimulus to compensatory processes than in the larger vessels. It is possible, therefore, that successive musculo-elastic layers are formed more rapidly in the smaller vessels and that the rate of reduplication of elastic membranes exceeds the rate at which they degenerate and disappear.

In the larger vessels the development of the elastic hyperplastic layer is associated with the onset of medial fibrosis and the period of decline in arterial development. Many believe that the formation of this layer is secondary to degenerative changes in the underlying media (Thoma, 1883; Blumenthal, Lansing, and Gray, 1950; Crawford and Levene, 1953). The present study would support this view. Medial fibrosis was found to be most marked in those areas of longitudinal hypertrophy thought to be subject to haemodynamic stress. Over these regions the elastic hyperplastic layer tended to become nodular and, in the later decades, atheromatous. In this way, many foetal cushions mark the site of a future atherosclerotic plaque, and, as Morgan (1956) has shown, atherosclerosis becomes common in the popliteal as well as in the coronary arteries.

The influence of pulsatile stresses in causing the localization of atherosclerosis at the mouths of branches has been stressed by Duguid (1926). It is believed that the muscular cushions of the foetus and the later development of the peripheral arteries traced in this study confirm the presence of such a stress and indicate its importance in the localization of intimal plaques.

I wish to thank Professor J. H. Biggart for his help and advice during the course of this work.

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ANGIOSARCOMA OF THE HEART

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The clinical features and pathological details of a primary cardiac angiosarcoma are described. The significance of the various clinical manifestations of primary cardiac tumours is discussed. The incidence, the histological diagnosis, and the histogenesis of primary cardiac angiosarcoma are considered.

The heart is rarely the site of neoplastic diseases. Lymburner (1934) found 56 cardiac tumours in 8,550 necropsies, Scott and Garvin (1939) 79 cases in 11,100 necropsies, Reisinger, Pekin, and Blumenthal (1942) an incidence of 1.7% in 355 necropsies, Lange and Christiansen (1947) 40 cardiac neoplasms in 4,915 necropsies, and Amsterdam, Grayzel, and Louria (1949) 19 cases in 5,000 necropsies. In this hospital seven cardiac tumours were found in a series of 1,643 necropsies.

Primary cardiac neoplasms are rarer than secondary tumours. Different proportions of primary to secondary growths have been reported by various workers. Lymburner (1934) recorded one in 13, Lange and Christiansen (1947) one in 39, Amsterdam *et al.* (1949) one in 18, and Cheng and Sutton (1955) one in 16. In the cases collected from this hospital the proportion was one in seven.

Mahaim (1945) reviewed 329 primary cardiac tumours. Eighty-seven of these growths were sarcomata, of which three were angiosarcomata. A search of the literature has revealed 13 more examples of angiosarcomata, thus giving an apparent total of 16 cases, details of which are set out in Table I. It is evident that primary cardiac angiosarcomata are very rare tumours. It is considered, therefore, that it will be of interest to record another case.

Case Report

Clinical History.—J. C., a woman aged 52 years, had had good health until December 3, 1957, when she developed a pain in the chest which she thought was due to a cold, but she continued with her work as a part-time cleaner until December 7. The next day, apart from the chest pain she felt well. In the afternoon she complained of a pain in the abdomen, but she continued with her housework. On December 9 she felt unwell and stayed in bed, but did not feel ill enough to send for her doctor. Two days later, as she was no better, she treated herself by taking a proprietary fever medicine. On the afternoon of December 13 her condition worsened and her doctor was summoned. He found her seriously ill. She died in the ambulance on her way to hospital.

Necropsy.—The necropsy was done 20 hours after death.

Both pleural cavities contained a small quantity of straw-coloured fluid. Mucopus was present in the trachea and bronchi. The lungs were oedematous. The liver had a vivid nutmeg pattern. The left ovary contained small simple cysts and the uterus was atrophic. The spleen, pancreas, adrenals, alimentary and urinary tracts, brain, and skeletal systems showed no abnormalities. Metastases were not seen in any of the above organs. The pericardial sac was distended with blood.

Heart.—The heart weighed 665 g.

Most of the wall of the right atrium was replaced by a purple tumour. It measured 7 cm. by 4.5 cm. by 5 cm. and extended upwards to involve the auricular appendage and backwards almost to the site of pericardial reflexion. Its external surface was irregular and the cut surface showed an outer rim of tissue, 0.5 cm. to 1 cm. thick, which enclosed a central core of blood clot. The neoplastic material had a spongy appearance, but in the inner portion it was more compact and formed a thin capsule around the blood clot (Fig. 1). The endocardium of the atrium was smooth and pale apart from a few areas on its lateral wall where the purple tumour lay beneath it. Numerous flat or hemispherical nodules were scattered over the epicardial surface of the ventricles and the inner surface of the pericardium (Fig. 1). The largest one was situated on the left ventricle just in front of the auricular appendage and measured 2 cm. by 2.5 cm. by 2.5 cm. The cut surface of these nodules had a similar appearance to that of the main growth. The cardiac valves were normal. Neither the cardiac valves nor coronary arteries showed any connexion with the neoplastic process.

TABLE I

SUMMARY OF REPORTED CASES OF ANGIOSARCOMA OF HEART

Author	Age (Yr.)	Sex	Clinical Features	Site of Origin	Metastases
Menning (1888) quoted by Mahaim (1945)	18	F	Haemopericardium	Right auricle	Pericardium
Gödel (1922)	44	M	Pericardial effusion	,, ,,	Pericardium, lungs, brain
Hewer and Kemp (1936)	62	F	Cough, haemoptysis, paraplegia	,, ,,	Pericardium, bronchial lymph nodes, omentum, bones
Gross and Englenart (1937)	45	F	Old rheumatic endocarditis with mitral stenosis, left hemiplegia, congestive heart failure	Left ,,	None
Choisser and Ramsey (1939)	26	M	Congestive heart failure	Right ,,	Pericardium, pleura, liver,
	30	M	Pericardial effusion	,, ,,	omentum, pericardium
Weller (1940)	30	M	Cough, swelling of face and neck, pericardial effusion, hepatomegaly	,, ,,	None
	26	M	Influenzal attack, hepatomegaly, lymphadenopathy	,, ,,	Pericardium, pleura, lungs, peritoneum, spleen, liver, kidneys
Lange and Christiansen (1947)	47	M	Pericardial effusion	,, ventricle	Pericardium, pleura, lungs, mediastinal lymph nodes
Amsterdam *et al.* (1949)	54	M	Congestion of face and veins of neck, bilateral pleural effusions, congestive heart failure	Infundibular cusps of the tricuspid valve	None
Glassy and Massey (1950)	26	M	Congestive heart failure, precordial pain, dyspnoea	Right auricle, or right ventricle	Pericardium, pleura, lungs
Tacket *et al.* (1950)	45	F	Congested veins of neck, swollen face, tachycardia, pericardial effusion, hepatomegaly	Interauricular septum of right auricle	Pericardium, lungs
Blanchard and Hethrington (1952)	25	M	Cough, precordial pain, cyanosis of head, neck, and chest, pleural and pericardial effusion, hepatomegaly	Right auricle	Pericardium, lungs, liver, adrenals
	33	M	Precordial pain, dyspnoea, bilateral pleural effusions, pericardial effusion	,, ,,	Pericardium, lungs, liver, bones
Schwartz and Loder (1952)	37	M	Precordial pain, dyspnoea, pericardial effusion	,, ,,	Pericardium, pleura, lungs, spleen
Cheng and Sutton (1955)	45	M	Cough, haemoptysis, precordial pain, dyspnoea, swelling of face and feet, pericardial effusion	,, ,,	Pericardium, lungs, liver

Histology.—Several blocks of tissue were taken from the tumour on the wall of the right atrium and the nodules on the epicardial surface of the ventricles and pericardium. Sections prepared from these blocks were stained with haematoxylin and eosin, Mallory's phosphotungstic acid haematoxylin, Heidenhain's haematoxylin and eosin, Heidenhain's haematoxylin combined with Masson's trichrome stain, and Gordon-Sweet reticulin stain counterstained with haematoxylin and eosin.

The neoplastic tissue was composed of spindle, round, cubical, or oval cells and their nuclei were round or oval and vesicular in type (Fig. 6). Some nuclei were hyperchromatic and an occasional mitotic figure was seen. The arrangement of the cells varied in different portions of the growth. In the outer areas of the tumour they formed a loose network in which small vascular channels were apparent (Fig. 2). These channels became larger towards the centre of the growth and were separated from one another either by a considerable amount of cellular tissue or by less cellular strands (Figs. 3 and 4). The vascular spaces contained red blood cells and were lined by flattened or cubical cells (Figs. 3, 4, 5, and 6) and in scattered foci these cells were heaped up on each other (Figs. 5 and 6). Reticulin-stained sections showed that there was a free anastomosis between the vascular channels (Fig. 7). Areas of haemorrhage and necrosis were present in the inner zones of the growth, which obliterated the blood spaces or filled them with polymorphonuclear leucocytes. Infiltration and destruction of the myocardium by the neoplastic tissue was seen in the outer portions of the growth (Figs. 2, 5, and 7).

The nodules on the epicardial surface of the ventricles and pericardium had a similar histological structure to that of the tumour of the right atrium (Fig. 8).

Discussion

Mahaim (1945) considered that persons who had benign pedunculated cardiac neoplasms might be cured by operative procedures. It is important, therefore, to attempt to diagnose these tumours in life. Yater (1931), Mahaim (1945), and Whorton (1949) studied the clinical features of these growths. They showed that unremitting heart failure of obscure origin, precordial pain, cardiac arrhythmias, signs of obstruction of the superior

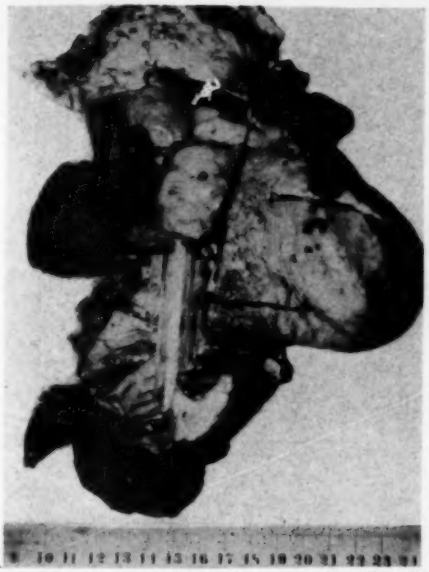

FIG. 1.—The heart with the right atrium opened and small portion of the pericardium to show the primary tumour in the right atrium and secondary nodules on the epicardial surface of the ventricles and pericardium.

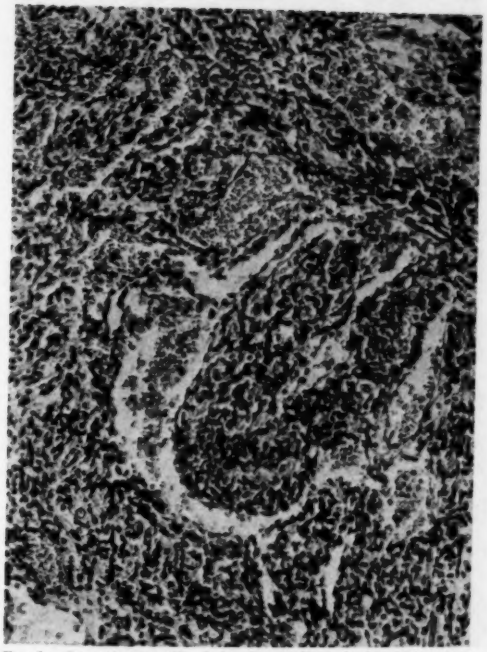

FIG. 3.—Same tissue as Fig. 2 showing medium-sized vascular spaces containing red blood cells, lined by flattened or cubical cells and separated by a considerable amount of cellular tissue. Haematoxylin and eosin. × 180.

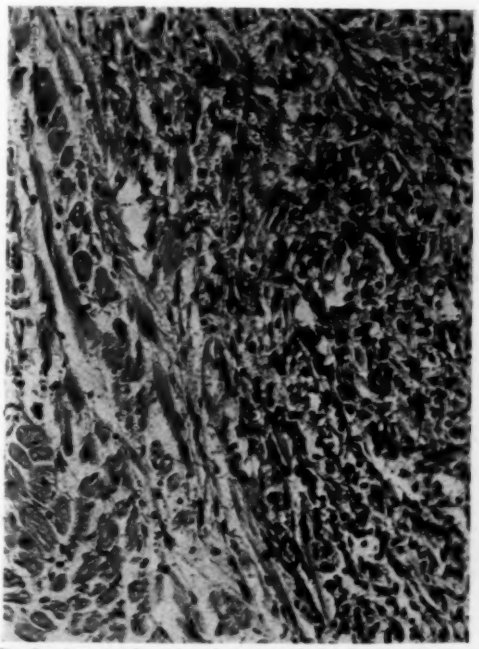

FIG. 2.—A section from the primary growth showing extension into the myocardium and the loosely interlaced neoplastic cells forming small vascular spaces. Haematoxylin and eosin. × 180.

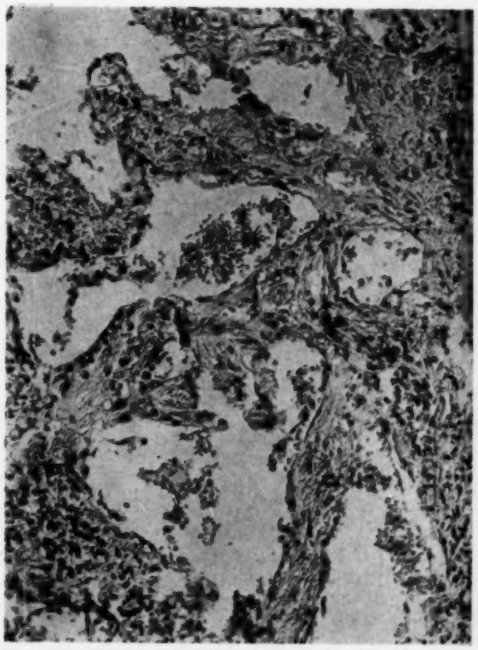

FIG. 4.—Same tissue as Fig. 2 showing large vascular channels containing red blood cells, lined by flattened or cubical cells and separated by smaller strands of cellular tissue. Haematoxylin and eosin. × 180.

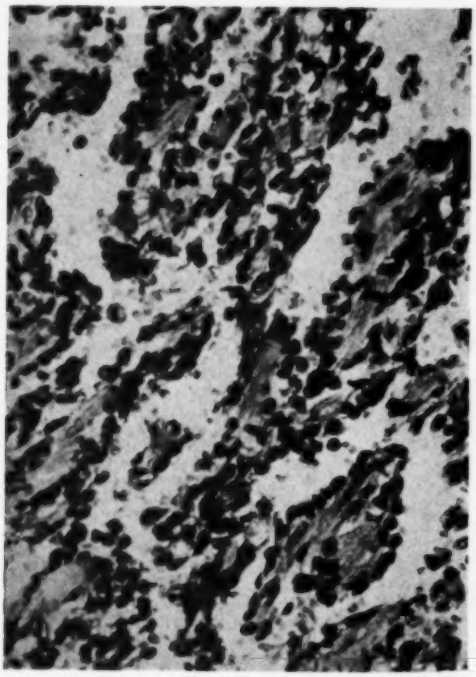

FIG. 5.—Same tissue as Fig. 2 showing vascular spaces lined by flattened or cubical cells, some of which are heaped up on each other and muscle fibres infiltrated by the neoplastic cells. Haematoxylin and eosin. × 325.

FIG. 7.—Same tissue as Fig. 2 showing anastomosing vascular channels, which infiltrate muscle fibres. Gordon-Sweet reticulin stain. × 325.

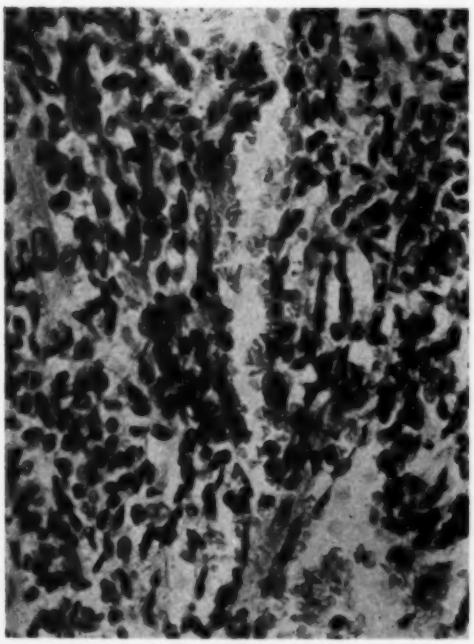

FIG. 6.—Same tissue as Fig. 2 showing spindle, round, cubical, or oval cells with round, oval, and vesicular nuclei and vascular channels lined by flattened or cubical cells. Haematoxylin and eosin. × 410.

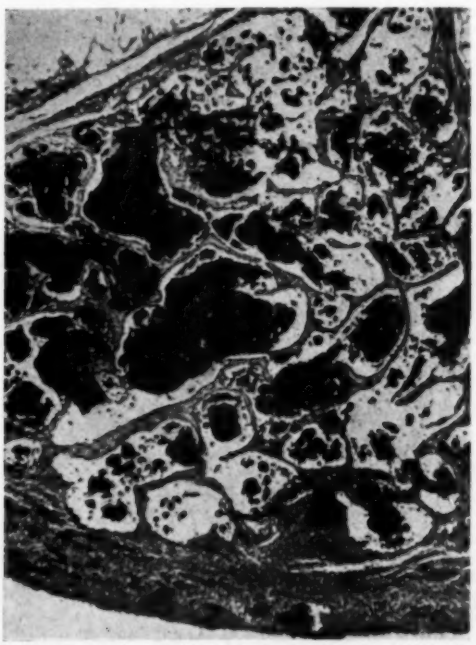

FIG. 8.—Section from nodule on pericardium showing anastomosing vascular spaces containing red blood cells and separated by a variable amount of neoplastic tissue. Haematoxylin and eosin. × 29.

mediastinum, haemopericardium, non-pulsating enlargement of the right auricle, and sudden death could be caused by cardiac tumours. The clinical features presented by previously reported cases of angiosarcoma of the heart (Table I) show that these tumours may evince any of the above clinical manifestations except sudden death. Thus it is apparent that there is no special clinical feature which will distinguish between benign and malignant cardiac tumours. Contrariwise, a haemopericardium indicates that the neoplasm is either a primary cardiac tumour which has spread to the pericardium, or one which is arising from the pericardium, and is not a benign pedunculated cardiac growth. It is interesting that in the case reported above the large cardiac tumour gave rise to no clinical manifestations until 10 days before death, and probably these were caused by the cardiac tamponade.

Evans (1956) has pointed out that angiosarcomata should be differentiated from mesenchymomas which show vasoformative and other lines of mesenchymal differentiation. In this case special staining procedures failed to demonstrate any line of mesenchymal differentiation other than vasoformative ; thus this tumour cannot be considered to be a mesenchymoma with vasoformative tendencies.

In this case the diagnosis of an angiosarcoma was made on the criteria laid down by Stout (1943). He considered that these tumours were composed of atypical endothelial cells, which were formed in greater number than required to line vascular spaces by a single layer of endothelium. These channels formed an anastomosis with each other and were surrounded by a delicate framework of reticulin. The neoplastic cell varied from a polygonal to a spindle-shaped cell and could form a single layer, heaps of cells within the vascular lumen, or sheets of cells outside the vascular channel. All these features were evinced by the tumour described above.

Since the site of origin of primary cardiac angiosarcoma is the wall of the heart, the angiosarcoma arising from a coronary artery described by Schwarzkopf and Gais (1953) and the angiosarcomata originating from the pericardium reported by Greenberg and Angrist (1948), Florange (1954), and Grosse-Brockhoff and Schreiber (1955) have not been included in the list of published cases of primary angiosarcoma of the heart noted in Table I.

McClure (1921) has shown that primitive mesenchymal tissue in any part of the body can form angioblasts. Primary cardiac angiosarcoma thus probably arise from these cells which normally lie dormant in the myocardium. This is in agreement with the opinions expressed by Straus and Merliss (1945) and by Tacket, Jones, and Kyle (1950), who considered that primary cardiac tumours arose from mesenchyme. The latter authors observed that most of these tumours grew from the septal area of the heart, which is the last zone for complete structural formation. They considered, therefore, that this site of origin was corroborative evidence of the mesenchymal origin of primary cardiac tumours.

I wish to thank Dr. R. T. Cooke, who performed the necropsy, for permission to publish this case and for his helpful criticism, Dr. E. K. Dawson for her help and encouragement, Professor Duguid, who granted me facilities for photomicrography, and the Medical Photography Unit of Edinburgh University for Figs. 5 and 7.

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INCREASED MITOTIC ACTIVITY IN THE LIVER AND OTHER ORGANS IN A CASE OF ACUTE RENAL TUBULAR NECROSIS

BY

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A case is presented in which numerous mitotic figures were found in the liver, pancreas, and adrenal glands of a patient who died in uraemia due to acute tubular necrosis.

The possibilities of the failure of excretion of a waste product acting as a stimulus to mitosis, or that a mitotic stimulant is released from the acutely ischaemic kidney, are suggested.

The mitotic index of the adult mammalian liver is low (Brues and Marble, 1937; Swann, 1958). Increased mitotic activity is seen in the liver in cases of acute hepatic necrosis, and in the experimental animal mitotic division is a prominent feature in the liver remnant after partial hepatectomy (Higgins and Anderson, 1931).

Bullough (1950) has clearly demonstrated in epidermal cells in the mouse that no cells enter prophase after the animal's death, but that cells which have begun dividing in the living animal will complete their division. If this were true for the human liver cell, one would expect to find no mitoses in the liver at necropsy except in cases where the examination was performed shortly after death. However, mitotic figures are seen after death in cases of acute hepatic necrosis and occasionally they are seen in livers in which morphological evidence of liver damage is absent.

Case Report

L. T., a Bantu boy aged 15 years, was admitted with a history of frontal headache and unproductive cough for four days. Six days previously he had experienced nausea associated with abdominal pain, but this had resolved spontaneously. He had had no diarrhoea. The only other important point in the history, obtained on direct questioning, was that he had last passed urine three days before admission.

On examination, the patient was well nourished and did not appear to be acutely ill. The blood pressure on admission was 120/80 mm. Hg. No abnormality was detected in the cardiovascular or respiratory systems. The liver was palpable 1 in. below the right costal margin. Systemic examination revealed no further significant findings.

The patient passed a small quantity of urine shortly after admission and this contained a moderate amount of albumin and numerous granular, epithelial, and red cell casts.

The clinical diagnosis was acute glomerulonephritis and he was treated with antibiotics and maintained on a restricted oral fluid intake.

On the second morning after admission he had passed 6 oz. urine during 24 hours and his blood pressure was 130/90 mm. Hg. Shortly after being seen he had a generalized epileptiform convulsion and died.

Necropsy Findings

Necropsy was performed 24 hours after death.

The body was that of a well-nourished Bantu male (height 5 ft. 6½ in.).

The heart weighed 320 g. but showed no further abnormality. There was an acute tracheobronchitis with a resolving bronchopneumonia in the lower lobe of the left lung.

The kidneys weighed 260 and 280 g. The capsules stripped easily, leaving smooth, pale surfaces. The cortices were swollen and pale and the vascular markings were indistinct. Histologically both proximal and distal convoluted tubules were dilated and the lining epithelium was flattened. These changes were most prominent in the distal convolutions. Scattered cells in the distal convolutions and in the loops of Henle showed degeneration and necrosis. Focal tubulorrhexis was present. The tubular lining epithelium showed widespread signs of regeneration (Fig. 1). Many distal convoluted and collecting tubules and many of the loops of Henle contained red granular and haemoglobin casts and desquamated, sometimes necrotic, epithelial cells. Numerous names have been suggested for this lesion, but acute tubular necrosis seems to be the

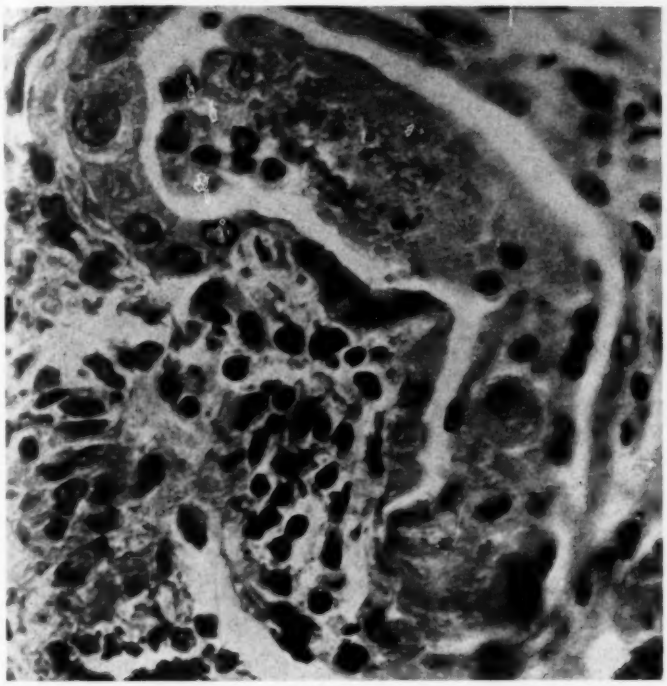

FIG. 1.—Dilated distal convoluted tubules in the kidney, containing granular casts and showing evidence of regeneration of the epithelium. Haematoxylin and eosin ×1,000.

FIG. 2.—Section of the liver showing a large [multinucleate [liver cell. Haematoxylin and eosin ×1,000.

FIG. 1

most satisfactory one at present (Sevitt, 1959). The aetiology of the lesion is uncertain in this case. (No primary glomerular lesion was noted.)

The liver weighed 1,860 g. and appeared macroscopically normal. On histological examination an occasional bilharzial granuloma was seen. The lobular pattern was preserved and the portal tracts and small bile ducts showed no abnormality. Mild to moderate nuclear polyploidy was present and there were many binucleate cells as well as a moderate number of tri- and multinucleate liver cells (Fig. 2). Numerous mitoses were distributed through-

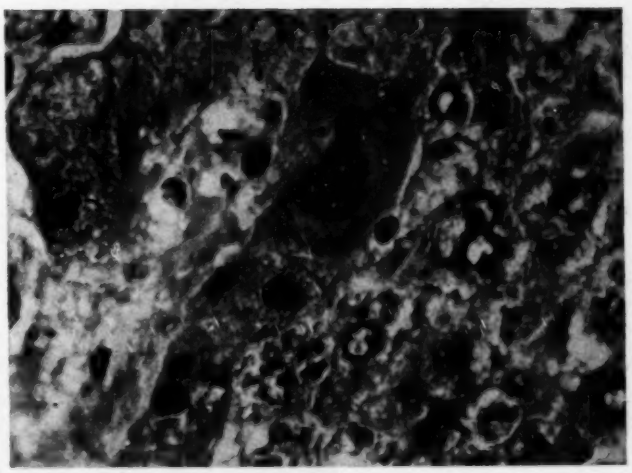

FIG. 2

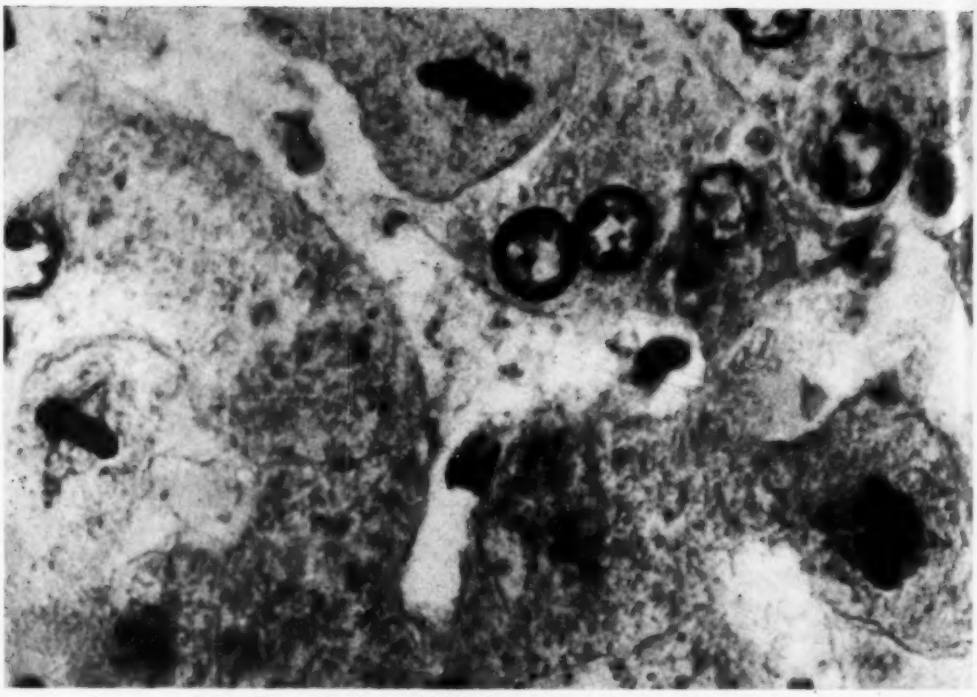

FIG. 3.—Three mitoses in a small field in the liver. Haematoxylin and eosin ×2,500.

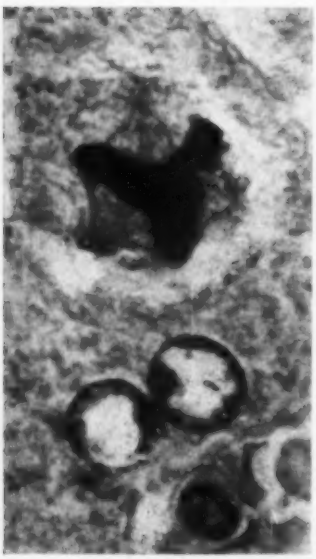

FIG. 4.—Section of liver showing a large tripolar metaphase. Haematoxylin and eosin ×2,500.

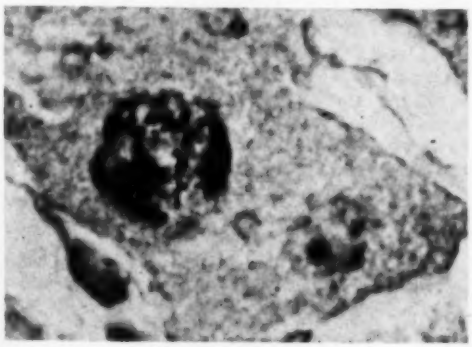

FIG. 5

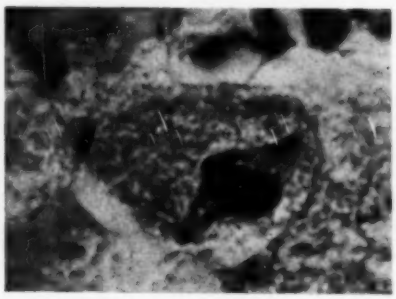

FIG. 6

FIGS. 5-7.—Liver cell nucleus in prop
(Fig. 5), in metaphase (Fig. 6),
in anaphase (Fig. 7). Haemato
and eosin ×2,500.

out the liver lobules (15/1,000 liver cell nuclei (Fig. 3) in counts of tissue sections). Several abnormal mitoses were present (2–3%) and those mostly took the form of tripolar spindles (Fig. 4). Some of the metaphases were large and suggested polyploid cells in mitoses. All phases of the cycle were represented, but metaphases predominated (Figs. 5–7), the proportions being prophase 5%, metaphase 80%, anaphase 13%, and telophase 2%.

The spindles in metaphase and anaphase were easily visible in sections stained with haematoxylin and eosin, but appeared otherwise morphologically normal. Neither fat nor glycogen could be demonstrated in suitably prepared and stained sections of liver.

Some mitotic figures were seen in the acinar and islet cells of the pancreas and in the cortex of the adrenal gland, chiefly in the zona fasciculata.

Discussion

Whether the mitotic figures represented a true increase in mitotic activity or a delay in the mitotic cycle is difficult to say on histological grounds alone. Complete inhibition of the mitotic cycle was unlikely as all phases are represented. A phase count on a liver biopsy from a case of viral hepatitis, in which many mitotic figures were present, showed a similar distribution (prophase 6%, metaphase 80%, and anaphase 14%). Also the presence of tri- and multinucleate liver cells suggested a true increase in mitotic activity.

Paget (1954) has shown that in post-mortem liver sections, excluding those from cases of liver disease, mitoses are seen almost exclusively in patients dying in acute or subacute uraemia. Bywaters (1946) found mitoses in the liver in 10 of 42 cases of traumatic anuria, and in two of the cases no evidence of necrosis was seen in the liver.

As a result of the striking mitotic activity in this case and the report by Paget, we are at present reviewing a consecutive series of post-mortem liver sections and the livers of 50 cases of acute tubular necrosis. Preliminary results confirm Paget's findings and show a correlation between mitotic activity in morphologically normal livers and the renal lesion in cases of acute uraemia (acute tubular necrosis). In attempting to explain the association with acute and not chronic uraemia, Paget felt that necrosis may be the important factor. Our case did not show extensive cellular necrosis, and necrosis is frequently not a marked feature in this type of renal lesion (Brun and Munck, 1957). Bywaters suggested that the mitoses represented a reparative response to functional damage to liver cells as a result of anoxia. Acute renal ischaemia appears to be the underlying factor in the production of the renal lesion (Sevitt, 1959). Although this is associated in many cases with a generalized state of shock, vasoconstriction and ischaemia are often more marked in the kidney than elsewhere in the body (Lauson, Bradley, and Cournand, 1944). Local anoxia may, therefore, not always be a marked feature in the liver.

Our case showed mitoses in the pancreas and adrenal cortex, and Bywaters found mitoses in other organs in two of his cases (kidney and pancreas in one and kidney and thyroid gland in another). This suggests the possibility of a humoral effect. It has been suggested (Swann, 1958) that the stimulus for mitosis in the remaining kidney after unilateral nephrectomy is the failure of excretion of a waste product and a similar mechanism may operate in acute renal shutdown. On the other hand, the common denominator in this type of case may be the renal lesion itself (acute tubular necrosis). It could then be postulated that either an inhibitor is no longer formed or a specific substance is released from the kidney in acute renal ischaemia which stimulates mitotic division in the liver and other organs, including, when the acute vasoconstrictive phase has passed, the renal tubular epithelial cells.

Further work on this problem may elucidate one of the mechanisms involved in mitotic stimulation or inhibition.

We wish to thank Professors J. Barnetson and B. J. P. Becker for reading the manuscript, and Mr. G. J. de Swardt for taking the photomicrographs.

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A HAEMORRHAGIC DISORDER IN PREGNANCY DUE TO AN "ANTICOAGULANT" PREVENTING THE CONVERSION OF FIBRINOGEN TO FIBRIN

BY

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A bleeding disorder occurring during labour and affecting mother and foetus is described.

All stages of coagulation were normal until the reaction fibrinogen→fibrin. Either there was some abnormality present in the fibrinogen molecule, or there was an "anticoagulant" acting at this stage.

The abnormality was reversible by protamine sulphate and toluidine blue, but in other respects did not resemble hyperheparinaemia.

Reversibility of a clotting defect by protamine or toluidine blue is not sufficient evidence on which to make a diagnosis of hyperheparinaemia.

Demonstration of a prolonged clotting time occurring in late pregnancy does not necessarily justify the diagnosis of "afibrinogenaemia."

Severe haemorrhagic disorders occurring towards the end of pregnancy have usually been found to be associated with hypo- or afibrinogenaemia (Dieckmann, 1936; Moore, 1954). A few cases of haemorrhagic disorder associated with the presence of an anticoagulant have also been described following pregnancy; for example, the cases described by Dreskin and Rosenthal (1950) and by Biggs and Macfarlane (1957), in which the anticoagulant concerned inactivated anti-haemophilic globulin.

This report concerns a fatal haemorrhagic diathesis which developed in an otherwise healthy woman in the 38th week of pregnancy. The disorder was at first thought to be due to afibrinogenaemia, but on further investigation it was shown to be due to the presence of an "anticoagulant" which prevented the conversion of fibrinogen to fibrin.

Methods

The following investigations were carried out by the methods described by Biggs and Macfarlane (1957): Bleeding time (Ivy), Hess test, whole blood clotting time (Lee and White), recalcification clotting time, one-stage prothrombin time using human brain thromboplastin (Quick), two-stage prothrombin time, and thromboplastin generation test.

Haemoglobin estimation, white cell and platelet count (direct), and thrombin generation tests were carried out by the methods described by Dacie (1956). Fibrinogen estimations were performed by the micro-Kjeldahl method.

Human "heparin" was prepared from liver obtained at necropsy performed half an hour after death. The method followed was the same as given in the British Pharmacopoeia (General Medical Council, 1948) except that cetavlon was employed instead of brucine in the final purification. This resulted in a preparation which had definite anti-coagulant properties which were reversible by protamine sulphate and toluidine blue. A further study of the properties and composition of this substance is being undertaken.

Case Report

A 35-year-old married woman, at term with her fifth pregnancy, was admitted to the obstetric department of the Christian Medical College Hospital. Her four previous pregnancies had all been uneventful. There was no history of any previous bleeding episode, and her menstrual periods had always been normal. The patient had had no serious illness in the past, and had not had any drugs or medicine recently.

Physical examination revealed an apparently healthy woman. The blood pressure was 100 mm. of mercury systolic and 60 mm. diastolic. The fundus was at 38 weeks' height, and the foetal head was floating with the vertex presenting. Uterine contractions were occurring once every five to eight minutes. The foetal heart sounds were normal and the rate was 144 per minute.

The patient's haemoglobin was 7.2 g. per 100 ml., and the blood film showed a moderate degree of hypochromia of the red cells. Platelets were present in the blood film in normal numbers. The urine contained a trace of albumin, but was otherwise normal.

The patient remained well for the next 48 hours after admission and had no further uterine contractions. On the evening of the second day after admission she developed fever with a temperature of 102°, and complained of mild headache. Clinical examination at this time revealed no cause for this fever, except that a centrifuged deposit of the urine contained 20–30 pus cells per high-power field. A blood examination showed a white cell count of 16,000 per c.mm. (82% polymorphs, 3% eosinophils, and 15% lymphocytes). The patient was given 10 grains of aspirin, and intramuscular penicillin therapy (600,000 units of procaine penicillin 12-hourly) was begun. On the morning of the third day at about 7.30 labour started again. At 10 a.m. the foetal heart sounds were noted to be irregular, and liquor amnii heavily stained with meconium was passed. By 3 p.m. the cervix was three-fifths dilated, but the foetal heart could no longer be heard. At this stage it was noted that the patient had a little bleeding from the gums, but the full significance of this was not realized. At 4 p.m. the bleeding from the gums had increased, and it was also noted that the patient was bleeding from a venepuncture wound, and that there was a moderate amount of bleeding per vaginam. Blood was taken for various coagulation investigations, and a blood transfusion with freshly drawn bank blood was started. At this stage it was suspected that this was probably a case of afibrinogenaemia, and this was apparently confirmed by the demonstration of a prolonged whole blood clotting time. As no fibrinogen or concentrated plasma was available, the only possible treatment was the transfusion of fresh blood. However, in spite of transfusion bleeding continued.

By 5 p.m. it had been shown *in vitro* that the blood, which was apparently incoagulable, could be made to clot by the addition of protamine sulphate. This suggested a diagnosis of hyperheparinaemia, and therefore an injection of 150 mg. of protamine sulphate was given intravenously as a 1% solution at 5.12 p.m. Unfortunately this was all the protamine sulphate available in the hospital and no more could be obtained immediately. Three minutes after the injection a further specimen of blood was taken for coagulation studies. Following the injection bleeding appeared to stop for about 20 minutes, but after this bleeding from the vagina, the gums, and various needle puncture wounds recurred. Blood transfusion was continued while further efforts were made to obtain protamine sulphate or toluidine blue, but unfortunately neither was readily available.

At 7 p.m. a stillborn foetus was delivered, and blood was taken from the umbilical vein for examination. At 7.15 p.m. the placenta was expelled and the bleeding from the vagina increased in severity. An intravenous injection of ergometrine was given, but it made little difference to the severity of bleeding. Blood transfusion was continued throughout, at times under pressure, in an endeavour to keep up with the blood losses. At 7.45 p.m. a solution of toluidine blue, 1 g. in 500 ml. of 5% glucose, became available, and was started as a rapid intravenous drip via a second vein. However, in spite of all therapy the patient's condition rapidly deteriorated, she developed peripheral circulatory failure and pulmonary oedema and died at 8.15 p.m. At the time of death it was noticed that the most recent blood from the vagina was clotting spontaneously. This was the first time that this had been noted. Immediately after death, blood was taken from the heart for further tests.

Post-mortem Examination

Post-mortem examination of the mother showed bluish staining of all the tissues in the body. This was most marked on the internal surface of the aorta, the inside of the renal pelves and ureters, and in the brain.

The trachea and bronchi contained a lot of blood-stained frothy fluid. There were some petechial haemorrhages in the wall of the aorta, otherwise the cardiovascular system was normal. There was some altered blood in the stomach, with patchy areas of petechial haemorrhage all over the mucosa. Both liver and spleen were congested, but otherwise normal. The kidneys were normal, except that there were numerous subcapsular petechial haemorrhages. The bladder also showed some small haemorrhages. The upper two-thirds of the body of the uterus was firm and well contracted, but the lower portion was soft and flabby with marked haemorrhage into the muscle. The placenta had been attached anteriorly at the junction of the upper and lower uterine segments. No abnormality was present in the central nervous system.

Post-mortem examination of the foetus showed no abnormality, except for atelectasis of the lungs, some congestion of the liver, and haemorrhage into the cortex of both adrenal glands.

Tissue sections from the various organs and from the skin, and bone marrow smears from both mother and foetus stained with toluidine blue failed to reveal any evidence of increase in the number of mast cells.

Investigation of Coagulation Defect in Maternal Blood

Except where otherwise specified investigations were performed on blood taken at 4 p.m. before any treatment had been given.

Bleeding Time.—Bleeding time at 4 p.m. was 15 minutes.

Hess Test.—The Hess test was negative at 4.10 p.m.

Platelet Count.—The platelet count was 60,000 per c.mm. This was confirmed by the examination of a stained blood film which showed a decrease in the number of platelets.

Bone Marrow.—The marrow fragments were slightly hyperplastic. The developing red and white cells and the megakaryocytes were normal.

Clotting Time.—The clotting time of whole blood was as follows:

Initial sample taken at 4 p.m. No clot formed after 24 hours. In a sample taken at 5.15 p.m., three minutes after 150 mg. of protamine sulphate had been given, a few threads of fibrin appeared after one hour.

In a sample taken immediately after death the clotting time was 10 minutes. This formed a good solid clot which gelled the whole of the blood, and retracted normally.

Effect of Addition of Protamine Sulphate in Clotting Time of Plasma.—The addition of protamine sulphate, 10 mg., to 1 ml. of uncitrated plasma resulted in the rapid formation of a network of fibrin. As the amount of protamine sulphate added was decreased, the time at which the fibrin appeared was prolonged, the amount of fibrin formed decreased, and the nature of the fibrin changed from a network of fibrin strands to a few fine granules of fibrin. When the amount of protamine added was sufficiently decreased no fibrin was formed at all (Fig. 1). Similar results were found with the addition of toluidine blue.

patient's plasma	1	1	1	1	1	1	1	1
protamine mgm.	0	10	5	2·5	1·2	0·6	0·3	0·15
clotting time seconds	∞	1	5	18	25	40	∞	∞
size of clot								

FIG. 1.—The clotting time and the size and nature of the clot produced, when varying amounts of protamine sulphate were added to the patient's uncitrated plasma. In each case the protamine was made up to 0.5 ml. with normal saline and added to 1 ml. of plasma.

Recalcification Time of Normal Plasma on Addition of Patient's Plasma.—The addition of the patient's plasma to normal plasma in equal volumes caused only a slight increase in the recalcification clotting time. As the amount of patient's plasma relative to normal plasma was increased the recalcification time became more prolonged (Fig. 2), and the size of the clot formed progressively decreased, suggesting that the fibrinogen from the patient's plasma did not contribute significantly to the fibrin formed.

Quick's One-stage Prothrombin Time.—The one-stage prothrombin time showed no clot after four hours.

control plasma	0	1	2	3	4	5	6	7	8	9	10
patient's plasma	10	9	8	7	6	5	4	3	2	1	0
clotting time seconds	∞	∞	615	205	190	175	170	165	155	150	150
size of clot											

FIG. 2.—The recalcification clotting time and the size and nature of the clot produced with varying mixtures of control and patient's plasma.

Two-stage Prothrombin Time.—As measured by the area under the graph, this gave a result equivalent to 92% of a normal control.

Plasma "Fibrinogen."—When the plasma was clotted by the addition of protamine sulphate, the amount of fibrin formed from fibrinogen was 210 mg. per 100 ml. of plasma.

Thrombin Generation Test. — Thrombin generation tests were carried out using the patient's platelet-rich plasma and a normal control plasma. The platelet count of the control plasma was adjusted by mixing "high spun" platelet-poor plasma with "low spun" platelet-rich plasma until the platelet count was approximately equal to that of the patient's plasma, viz., 120,000 per c.mm. The results of the thrombin generation test are shown in Fig. 3. It will be seen that the patient's plasma generated thrombin in a manner exactly comparable to normal control plasma with the same platelet count.

The results of the thrombin generation test on the patient's plasma were then compared with thrombin generation tests performed on the control

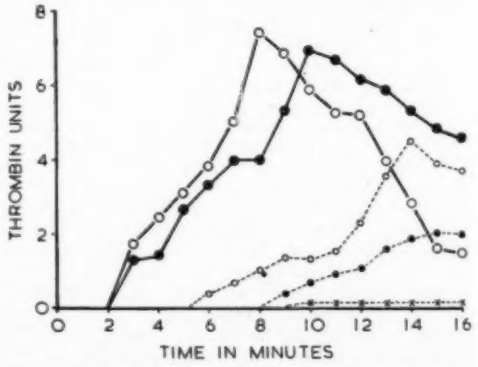

FIG. 3.—Results of thrombin generation tests: O———O Normal control. ●———● Patient. O - - - O Control + heparin—clotting time 20 minutes. ● - - - ● Control + heparin—clotting time 40 minutes. X - - - X Control + heparin—infinite clotting time.

plasma to which varying amounts of heparin* had been added. When sufficient heparin was added to the control plasma to make the clotting time greater than 12 hours no thrombin production could be demonstrated in the thrombin generation test. Decreasing the amount of heparin added caused increasing amounts of thrombin to appear, but the peak of the thrombin production was depressed and delayed (Fig. 3).

Similarly, when sufficient human "heparin" was added to the control plasma to render it incoagulable, there was no detectable thrombin formation in the thrombin generation test.

Thromboplastin Generation.—The thromboplastin generation test was carried out using the patient's barium sulphate adsorbed plasma, "serum" obtained after the patient's uncitrated blood had been kept for 12 hours at 37° C., and a platelet suspension from a normal control. The

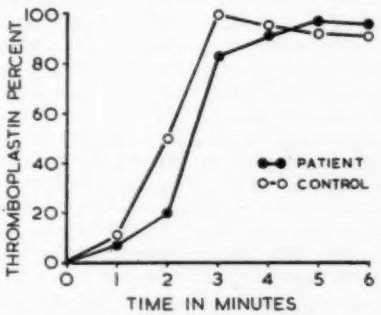

FIG. 4.—Results of thromboplastin generation tests. O——O Control. ●——● Patient.

results were identical with the thromboplastin generation when control-adsorbed plasma, control serum, and the same suspension of platelets were used (Fig. 4).

Fibrinolysin.—A clot from normal blood suspended in the patient's plasma did not undergo any detectable lysis when the two were incubated for 12 hours.

Attempted Transfer of the "Anticoagulant."—Protamine sulphate was added to the patient's plasma drop by drop until fibrin was no longer precipitated. The fibrin was then removed by centrifugation and nucleic acid added to the supernatant to combine with the protamine, and the precipitate so formed was then removed by centrifugation. The resultant "serum" was then added to normal citrated plasma and the recalcification time measured. The addition of this "serum" did not cause any significant prolongation of the clotting time of the normal plasma.

* "Boots" commercial heparin.

Investigation of Foetal Blood

Clotting Time.—No clot was formed after 24 hours. As with the maternal blood samples, the clotting defect was corrected by the addition of protamine sulphate and toluidine blue.

One-stage Prothrombin Time.—No clot was formed after four hours.

Further investigations on the foetal blood were not possible owing to the small amount of blood available.

Discussion

Circulating Anticoagulants in Pregnancy.—Several authors have reported haemorrhagic disorders associated with an anticoagulant occurring weeks or months after pregnancy, e.g., Madison and Quick (1945), Dreskin and Rosenthal (1950), Biggs and Macfarlane (1957). However, these cases had normal one-stage prothrombin times and had anticoagulants which appeared to act in the earlier stages of coagulation. The cases described in the latter two papers had an anticoagulant which was shown to be active against antihaemophilic globulin, and therefore differ from the present case.

Heparin-like Anticoagulants. — Jaques and Waters (1941) showed that a considerable rise in blood heparin occurred in dogs suffering from anaphylactic shock. Allen, Sanderson, Milham, Kirschon, and Jacobson (1948) found that the lengthening of the coagulation time which was seen in dogs exposed to ionizing radiation could be corrected by the addition of toluidine blue and protamine, and postulated that the cause of the haemorrhagic disorder was hyperheparinaemia. Castex (1946) and Castex and Pavlovsky (1947) reported a patient who had an anticoagulant which in some respects resembled heparin. Conley, Hartmann, and Morse (1949) described one patient in whom there was a heparin-like anticoagulant present in the plasma. Crisalli and Cotellessa (1951) described a 10-year-old boy with a haemorrhagic diathesis in which the clotting defect was corrected by toluidine blue and protamine. Bell (1951) reported a patient who had an anticoagulant which had both antithromboplastic and antithrombic activity, and was neutralized by the addition of protamine.

Physiological Action of Heparin.—Heparin interferes with the normal clotting mechanism in several ways. Howell and Holt (1918) found that the presence of heparin in the blood prevented the

conversion of prothrombin to thrombin, and concluded that heparin was an antiprothrombin. Mellanby (1934) found that heparin inhibited the thrombin-fibrinogen reaction, and Quick (1938) showed that a component from the plasma albumin was necessary for this inhibitory effect. Klein and Seegers (1950) showed that heparin promoted the adsorption of thrombin by fibrin. Lyttleton (1950) showed that heparin, in association with a fraction of the plasma albumin, combined with thrombin, thus reducing its ability to clot fibrinogen. He also showed that the combination of heparin and thrombin is reversed by toluidine blue. Biggs, Douglas, and Macfarlane (1953) showed that heparin inhibited the formation of blood thromboplastin and suggested that this is the reason for the failure of prothrombin conversion in heparinized blood.

Resemblance of Clotting Defect to Hyperheparinaemia.—It is interesting that although the clotting defect in our patient was reversed by toluidine blue and protamine, and so resembled hyperheparinaemia, in all other respects it differed in its action from the recognized properties of heparin. As shown by the thromboplastin generation test the formation of blood thromboplastin was not inhibited. Similarly the thrombin generation test showed that the "anticoagulant" did not interfere with the conversion of prothrombin to thrombin, or combine with thrombin to make it inactive. The normal thrombin generation test obtained with the patient's plasma contrasted markedly with the results of thrombin generation tests performed on normal plasma to which heparin had been added in sufficient amounts to render it incoagulable (Fig. 3). Moreover, this inhibition of thrombin formation was seen with both animal heparin and human "heparin."

The results of mixing the patient's plasma in varying amounts with normal plasma showed that the patient's plasma did not have the property of significantly prolonging the clotting time of normal plasma, except when the patient's plasma was present in large excess. This also is in marked contrast to blood to which heparin has been added in sufficient amounts to render it incoagulable, since such blood will cause considerable prolongation of the clotting time of normal blood.

Meneghini (1951) showed a correlation between the number of mast cells in the tissues and heparin activity of the blood in cases of leukaemia, liver disease, etc. In the present case there was no increase in the tissue mast cells either in the patient or the foetus.

In view of these findings it appears that, although the defect was reversible by protamine sulphate and toluidine blue, it was nevertheless not due to hyperheparinaemia. As a corollary it may be stated that reversibility by protamine sulphate and toluidine blue is not an adequate criterion for the diagnosis of hyperheparinaemia. Both protamine and toluidine blue are strongly basic substances which may react with many acidic macromolecules other than heparin.

Possible Site of Action of the "Anticoagulant."—Normally the conversion of fibrinogen to fibrin under the action of thrombin takes an appreciable time, and the action of thrombin is considered to be enzymatic in nature (e.g., Eagle, 1935; Morrison, 1947). Biggs and Macfarlane (1957) suggest that the formation of fibrin from fibrinogen involves three stages. First, acidic groups are split off from fibrinogen under the enzymatic action of thrombin. Secondly, the molecules thus formed polymerize, and thirdly under physiological conditions the fibrin so formed is strengthened by other linkages.

The fact that when protamine sulphate was added to the patient's uncitrated plasma in sufficient quantities fibrin appeared almost immediately suggests that the process of conversion of fibrinogen to fibrin under the action of thrombin had progressed at least part of the way, and that the "anticoagulant" was acting at some point in the reaction fibrinogen→fibrin, perhaps preventing the proper polymerization of the molecules.

The results shown in Fig. 2, where the addition of increasing amounts of protamine sulphate to the patient's plasma increased the size of the clot formed, suggest that there was a stoichiometric relationship between the protamine and its combination with the "anticoagulant."

Although the abnormality has been referred to as an "anticoagulant" the findings would be equally explicable on the basis of some alteration in the fibrinogen molecule itself, the abnormality being reversible by the addition of the strongly basic substances protamine sulphate or toluidine blue. The failure of the patient's plasma significantly to prolong the clotting time of normal plasma, and the fact that in the mixing experiments the amount of fibrin formed was proportional to the amount of normal plasma present (Fig. 2), are also in favour of this hypothesis of "altered" fibrinogen.

Frick (1955) described the occurrence of a plasma factor which inhibited the conversion of fibrinogen to fibrin in cases of multiple myelomatosis. In three cases the whole blood clotting

time ranged from 13 to 25 minutes, and the thrombin time was prolonged. On electrophoresis an inhibitor was found which migrated with the β and γ globulin fractions. The inhibitor had an effect on normal plasma, and its action was apparently not counteracted by protamine. It therefore appears that the inhibitor in these cases was different from the one described here.

"Afibrinogenaemia" and "Altered" Fibrinogen. — The commonest clotting disorder associated with pregnancy is the so-called "hypo" or "afibrinogenaemia" (Moore, 1954), and the occurrence of a prolonged clotting time in our patient first suggested the diagnosis of "afibrinogenaemia."

Recently it has been shown that so-called "afibrinogenaemia" may not necessarily be associated with low levels of fibrinogen (Sharp, Howie, Biggs, and Methuen, 1958). These authors studied four cases of this syndrome and found that in three cases plasma fibrinogen levels, as estimated biochemically, were not such as would usually be considered dangerously low (250, 148, and 298 mg. per 100 ml. respectively). As a result of their study, Sharp *et al.* suggest that in the early stages of this syndrome poor reactivity of fibrinogen to thrombin may be an important feature of the coagulation defect. These authors studied the effects of toluidine blue *in vitro* in their cases, and found it to be without effect. However, no mention is made of the concentrations used, or of any study of the effect of protamine sulphate. It is interesting that their cases also had thrombocytopenia.

Other Features.—It is noteworthy that the foetus was also affected in the same way as its mother, showing that the "anticoagulant" concerned could readily pass the placental barrier. This transplacental passage is perhaps further evidence that we are dealing with an actual anticoagulant rather than an abnormality of the fibrinogen molecule itself.

The presence of thrombocytopenia is an interesting though unexplained feature which may have contributed to the haemorrhagic state. It is interesting to note that Copley (1948) has suggested that the thrombocytopenia in subjects exposed to atomic bomb radiation may be due to increase in the blood "heparin." It may be that the thrombocytopenia in this patient was a secondary effect following on the presence of the "anticoagulant" and severe haemorrhage.

Treatment.—The tests *in vitro* suggested that protamine sulphate and toluidine blue would correct the abnormality. Unfortunately only a small amount of protamine was available and it took some hours to get toluidine blue. However, both these substances definitely improved the clotting mechanism. The patient died in the middle of the infusion of toluidine blue, but it is pure conjecture to speculate whether or not some reaction to the toluidine blue (which was only a laboratory stain and possibly not very pure) hastened her death.

The results of the mixing tests (Fig. 2) suggest that there was little or no excess "anticoagulant" free in the plasma, and it seems probable that the patient would have responded well to the administration of fibrinogen, or concentrated plasma had either of these been available. In the present state of our knowledge such a response might have been taken as a further pointer in favour of a diagnosis of "afibrinogenaemia."

We wish to thank Dr. D. Paranjothi, under whose care the patient was admitted, for asking us to see the patient and for permission to publish this report; Dr. E. Gault for the post-mortem findings and histological examinations; Miss M. Nash for the biochemical estimation of plasma fibrinogen; Dr. Ian Hansen for his help and advice; Miss I. Ponnuswamy for preparing the human heparin; and Mr. S. P. Swaminathan for technical help.

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ANTIFIBRINOLYTIC ACTIVITY IN NORMAL PREGNANCY

BY

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It is found that serum antifibrinolytic activity is inhibitory both to the spontaneous fibrinolytic activity and to the activity of streptokinase-activated plasminogen.

The titres of serum antiplasmin activity during gestation, labour, and the puerperium are all of the same order and do not differ from those in non-pregnant women. The marked decrease in the spontaneous fibrinolytic activity in late pregnancy and labour is therefore due to an actual reduction in the circulating active lytic enzyme and is not associated with a change in the antifibrinolytic activity.

The fibrinolytic activity of plasma depends upon the activation of the inert plasminogen into an active plasmin, and is brought about naturally by the action of activators in the circulating blood and in the tissues (Astrup, 1956a). It can also be induced by means of such substances as streptokinase, chloroform, and many others (Astrup, 1956b). In certain abnormal circumstances, e.g., after an injection of adrenaline (Biggs, Macfarlane, and Pilling, 1947) or in the post-operative period (Truelove, 1952), a considerable degree of fibrinolytic activity can be detected. Under normal conditions, however, plasma shows little, if any, spontaneous fibrinolytic activity (Macfarlane and Biggs, 1946) unless special precautions are observed. When blood is cooled down to 0° C. immediately after collection and assayed within 15 minutes, about 90% of people show spontaneous fibrinolytic activity (Fearnley and Tweed, 1953) though the degree of that activity is not very marked (Biezenski and Moore, 1958).

It was shown in a previous communication (Biezenski and Moore, 1958) that the spontaneous fibrinolytic activity of plasma in normal pregnancy, as assessed by the ice-cold technique, varies according to the stage of pregnancy. In the first 15 weeks of gestation the activity is present in about 95% of cases. This corresponds closely to the value obtained in non-pregnant women. As pregnancy progresses, however, there occurs a gradual reduction in incidence till in the last few weeks and in labour the activity can be demonstrated in only about 10% of cases. There is a rapid return to a normal incidence in the immediate puerperium. The assessments of the degree of fibrinolysis in patients where lysis was demonstrable have similarly shown a decrease in late pregnancy and in labour.

The plasma fibrinolytic activity depends further on the inhibitory action of the antifibrinolytic system. The presence in plasma of substances which inhibit lysis has been known for many years. More recently Norman (1958) postulated the presence of two antifibrinolytic factors and Ratnoff, Lepow, and Pillemer (1954) found in serum three specific substances inhibitory to plasmin.

Irrespective, however, of the mechanism of the inhibition or of the possible multiplicity of its components, the higher the degree of the total antifibrinolytic activity the lower the level of the lytic activity. The present study is an attempt to find out whether the decrease in the fibrinolytic activity in late pregnancy and labour is due to a high antifibrinolytic titre.

Method

The antifibrinolytic activity of blood is assessed by observing the effect of serum on plasmin in a fibrinogen-thrombin system. When plasma is recalcified all the spontaneous fibrinolytic activity is adsorbed on to the clot (Fearnley, 1953). Antifibrinolytic activity, however, remains unaffected as shown by Grob (1949), who found the same degree of antifibrinolytic activity in plasma as in serum.

The quantitative assessment is based on Shulman's observation that the amount of fibrinolytic enzyme inhibited is proportional to the amount of serum added (Shulman, 1952a). A series of dilutions of plasmin is acted upon by aliquots of serum. The alternative method of adding aliquots of plasmin to

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varying dilutions of serum (Bozzo, Piomelli, and Schettini, 1956) did not in my hands give uniform results and was abandoned.

It will be noted that observations on the fibrinolytic activity in pregnancy (Biezenski and Moore, 1958) were made by assessing the spontaneous lytic activity. This may well differ with regard to the inhibitory system from the plasmin activity. For example, Biezenski and Moore (1959) observed, as did also Jacobsson (1955a), that, in distinction from plasmin, spontaneous fibrinolysin does not attack fibrinogen. Therefore, a series of preliminary experiments was carried out which consisted in adding 0.1 ml. of serum to doubling dilutions of plasma containing proved spontaneous fibrinolytic activity. The activity was inhibited in all cases, showing that serum contains an inhibitor of the spontaneous fibrinolytic system.

Reagents.—The following were used:

Fibrinogen.—A solution of human fibrinogen was prepared by the method previously described (Biezenski and Moore, 1958). It was used fresh or freeze-dried and reconstituted in buffer to a concentration of 250 mg.%.

Veronal Buffer.—*p*H 7.35.

Thrombin.—Thrombin (Roche) was dissolved in water to a concentration of 10 units/ml.

Plasmin.—Plasminogen powder was prepared from human plasma fraction III* by Cliffton and Cannamela's modification (Cliffton and Cannamela, 1953) of Kline's method (Kline, 1953). The powder was weighed and activated by streptokinase ("varidase"†) by the method of Fishman and Kline (1956). The solution of plasmin was stored frozen at −10° C. in 1 ml. amounts in the concentration of 60 mg.%. It was thawed immediately before use and the dilutions were adjusted with buffer.

All nitrogen determinations were carried out by Conway's microdiffusion technique.

Technique.—Twelve plasmin dilutions of the original concentration of 60 mg.% were prepared by the addition of buffer: 1/30, 1/60, 1/90, 1/120, 1/150, 1/180, 1/210, 1/240, 1/270, 1/300, 1/330, and 1/360. Of the 1/30 dilutions, 0.1 ml. was placed in a 4 in.×¾ in. glass tube; 0.1 ml. of the 1/60 dilution was placed in the second tube and so on. Blood was obtained by venipuncture into a glass tube without any anticoagulant. Serum was separated from the spontaneously clotted blood by centrifugation for 15 minutes at 3,000 r.p.m. and diluted 1/20 with buffer. Then 0.1 ml. of the diluted serum was added to all the plasmin tubes which were incubated for 10 minutes in a 37° C. water-bath. (Prolongation of the incubation time up to half an hour did not appreciably alter the results.) At the end of 10 minutes 0.2 ml. of fibrinogen solution and 0.2 ml. of thrombin were added to each tube in quick succession. The mixtures clotted in no more than 30 seconds. The formation of the clot in each tube was checked and the tubes were left in the water-bath for exactly 30 minutes. The tubes were then inspected for the preservation of clot.

The antifibrinolytic titre of serum was expressed in terms of the lowest dilution of plasmin inhibited.

The results were uniform and no zonal phenomenon was observed. Control experiments with normal saline instead of serum resulted in the absence of clot in all the tubes.

All the pregnant and non-pregnant women examined were normal.

*Donated by the American National Red Cross and obtained through the courtesy of Mr. Tillman D. Gerlough, of E. R. Squibb and Sons, New Brunswick, N.J.

†Commercial Varidase (Lederle) obtained through the courtesy of Mr. Collins, of the Dublin Branch of the American Cyanamid Company, Lederle Laboratories.

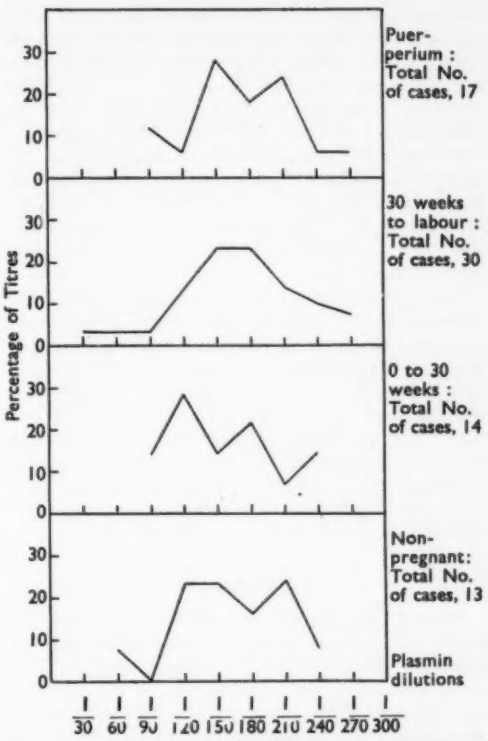
Puer-perium: Total No. of cases, 17
30 weeks to labour: Total No. of cases, 30
0 to 30 weeks: Total No. of cases, 14
Non-pregnant: Total No. of cases, 13
Percentage of Titres
30
20
10
0
Plasmin dilutions
30 60 90 120 150 180 210 240 270 300

FIG. 1.—Distribution of titres of antifibrinolytic activity in normal pregnancy.

Results

The patients were divided into four groups: non-pregnant, 0 to 30 weeks' gestation, 30 weeks' to labour inclusive, and the first week of the puerperium. In all, 74 patients were examined.

It can be seen from Fig. 1 that the distribution of titres within each group follows a fairly

symmetrical pattern. Each group presents a peak of incidence where most cases are concentrated with an even fall on either side of the peak. This marked concentration of titres is even more obvious when one realizes how small are the differences between successive plasmin dilutions. The range of titres is also very similar in all the groups, though the "30 weeks' to labour" group shows a slightly wider range than is seen in the other groups. This, however, is thought to be due to the larger number of determinations carried out.

Most titres of the non-pregnant series fall between the 1/120 and the 1/210 dilutions of plasmin; those of the "0 to 30 weeks' gestation" between 1/120 and 1/180 dilutions, as do those of the "30 weeks' to labour" group. The titres of the puerperal group fall between the 1/150 and the 1/210 dilutions. There is thus a marked similarity between all the groups which is further substantiated by the fact that the average titre value for each series is about the same, i.e., between the 1/150 and the 1/180 dilutions.

Discussion

Several reports on the antifibrinolytic activity in normal human plasma can be found in the literature (Shulman, 1952b; Bozzo *et al.*, 1956). It is interesting that irrespective of the method used they present similar results which are further confirmed by my findings. The differences in the level of the activity in normal people are insignificant though sporadic cases may show a marked increase. Furthermore, the activity is not influenced by sex or food ingestion and remains the same for the same individual for prolonged periods of time (Shulman, 1952a).

On the other hand, higher levels were reported in many abnormal states such as nephrosis (Jacobsson, 1955b), pernicious anaemia, pneumonia, and others (Guest, Daly, Ware, and Seegers, 1948), but in patients suffering from rheumatoid arthritis no departure from normal was noted (Hammarsten and Jonsson, 1954). Higher levels were found in "platelet-rich plasma" and lower than normal in thrombocytopenia (Stefanini and Murphy, 1956).

These observations show that the titre of antifibrinolytic activity throughout pregnancy, labour, and the puerperium is almost the same and, furthermore, does not differ from that found in non-pregnant women. Although it could be argued that inasmuch as uncomplicated pregnancy is a physiological process there is no reason for the antifibrinolytic activity to undergo any change, one must not forget that serum of pregnant women contains large amounts of substances which have been reported to affect the activity at least *in vitro* and in the animal. Thus Ungar and Damgaard (1951) found an increase in the antifibrinolytic activity in rats after injections of A.C.T.H. and cortisone, but their results were not confirmed in man by Cliffton (1952).

An increase in the antifibrinolytic activity after ingestion of butter and eggs was reported by Greig and Runde (1957). They attributed this effect to the high content of beta-lipoproteins in those foods, and it has been shown that beta-lipoproteins are much increased in pregnancy (Smith, de Alvarez, and Forsander, 1959).

The influence of high fibrinogen levels, as found throughout pregnancy, is not clear either. Shulman (1952a) reported that *in vitro* the fibrinolytic activity decreases with the increase in the amount of fibrinogen present whereas Norman (1958) found that in the fibrinogen-lysin-antilysin system the inhibitor competes with the fibrinogen substrate for the lytic enzyme so that with higher fibrinogen concentrations one would expect higher antifibrinolytic levels.

Since it is obvious from the results that there is no increase in the antifibrinolytic activity in late pregnancy and labour, the low titre of spontaneous fibrinolysis in those states must be due to an actual reduction in the circulating lytic enzyme. This may be due to an appearance in the plasma of those patients of an inhibitor which specifically inhibits the activation of plasminogen but which does not inhibit the active lytic enzyme and which, therefore, is not apparent in the experiments carried out. Or, again, it is possible that the plasma of women approaching term becomes deficient in that component of the lytic system which is responsible for the generation of spontaneous fibrinolytic activity.

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FIBRINOLYTIC AND ANTIFIBRINOLYTIC ACTIVITY IN PREGNANCY

BY

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Fibrinolytic activity and serum antifibrinolysin were estimated in normal pregnant women, during and after labour.

The decreased fibrinolytic activity found during labour returned to non-pregnant levels within 24 hours of delivery. During the same period, the serum antifibrinolysin was rapidly diminished.

It is suggested that the post-partum increase in fibrinolytic activity to non-pregnant levels is due to alterations in the fibrinolytic system itself, as well as to changes in circulating antifibrinolysin.

In a previous communication alterations in fibrinolytic activity during pregnancy were reported (Gillman, Naidoo, and Hathorn, 1959). It was shown that, within 13 to 15 weeks of the onset of pregnancy, fibrinolytic activity, as measured by euglobulin lysis time, was greatly reduced and remained so until the second stage of labour. Within 24 hours of delivery, there was a rapid increase in fibrinolytic activity, control levels being attained within 24 hours. These findings, using another method and in another racial group, were in agreement with the findings of Biezenski and Moore (1958).

However, the mechanism underlying these changes remains obscure. Whether the alteration in fibrinolytic activity during pregnancy is a function of fibrinolysin and/or precursors, antilysins, fibrin substrate, or a combination of these factors, is not known. To this end, serum antifibrinolytic activity was determined in nine pregnant African women, both during and after labour.

Materials and Methods

Pregnant African women in the first and second stage of labour were selected from the labour wards of the King Edward VIII Hospital. They were all medically healthy and obstetrically normal. In each of the women, determinations were made during labour and nine to 24 hours after parturition.

Collection of Blood.—The subjects were not under basal conditions. All blood samples were collected between 9 and 10 a.m. through a wide-bore needle inserted by clean venipuncture into the antecubital vein. The first few drops were discarded and 5 ml. of the freely flowing blood was collected in clean 10×70 mm. tubes containing 0.5 ml. of a 3.8% sodium citrate solution. The tubes were immediately placed in melting ice and transported to the laboratory.

Fibrinolysis.—Fibrinolysis was estimated within one hour of collecting blood by estimating euglobulin lysis time according to the method described by Von Kaulla and Schultz (1958). In essence this technique involves the isoelectric precipitation of euglobulin from citrated plasma, clotting with thrombin, and incubation at 37° C. The euglobulin lysis time is taken as the interval between the adding of thrombin and the complete dissolution of the clot.

Antifibrinolytic Activity. — This was estimated according to the method of Guest, Ware, and Seegers (1947). Serum was obtained by adding a drop of thrombin to citrated plasma and whipping out fibrin with a glass rod.

Results

It will be seen from Table I that, despite wide variation in age and parity, the euglobulin lysis time decreased in all nine patients to a median of 165 minutes which approximates to the normal non-pregnant level previously found by us (Gillman *et al.*, 1959). This decrease in euglobulin lysis time in all nine patients was statistically significant, and could only be expected by chance in less than one in a hundred patients ($P=0.01$).

There was a decrease in antifibrinolytic activity in eight of these patients, and no change in the remaining patient. This, likewise, was statistically significant ($P=0.01$).

TABLE I

EUGLOBULIN LYSIS TIMES IN NINE PATIENTS

Case No.	Age	Parity	Before Delivery			After Delivery		
			Hours	E.L.T. (min.)	Anti-lysin (units/ml.)	Hours	E.L.T. (min.)	Anti-lysin (units/ml.)
1	19	0	3	360+	437	21	210	200
2	18	1	1	360+	537	23	125	175
3	30	3	1	300	655	23	130	375
4	25	3	1	360+	600	23	130	250
5	32	0	5	180	650	20	165	525
6	19	1	13	242	715	11	165	525
7	28	3	3	360+	915	21	250	575
8	19	0	1	360+	500	23	225	500
9*	28	4	13	360+	550	11	100	535

* Twin delivery.

Discussion

Biezenski and Moore (1958) found that the proportion of pregnant women showing complete lysis of clots rose abruptly after delivery, indicating an increase in overall fibrinolytic activity. This finding was substantiated by Gillman *et al.* (1959), who demonstrated a marked decrease in euglobulin lysis time within the first 24 hours after birth.

Changes in overall fibrinolytic activity may result from alterations in fibrinolysin and/or its precursors, antilysins, fibrin substrate, or a combination of these factors. Plasma fibrinogen concentrations show no significant changes immediately following delivery (Gram, 1921 ; Plass and Matthew, 1926; Gillman *et al.*, 1959) and attention must thus be directed to the other components listed above.

In the present study, we have shown that, in addition to decreased euglobulin lysis time, there is also a significant decrease in antifibrinolysin following delivery. The question naturally arises as to whether this change in euglobulin lysis time may be partly or wholly due to the decrease noted in plasma antifibrinolysin.

Several reports in the literature indicate that antifibrinolysin is present mainly in the pseudoglobulin fraction of plasma proteins. Thus Macfarlane and Pilling (1946) could detect no plasmin-inhibiting activity in euglobulin precipitated at *p*H 5.5 and low ionic strength, whereas large amounts were present in the supernatant containing the pseudoglobulins. Milstone (1941), too, could detect inhibiting activity only in the pseudoglobulin fraction. More recently, Kowalski, Kopeć, and Niewiarowski (1959) found only traces of antiplasmin in euglobulin precipitates.

Thus in the euglobulin lysis time method of estimating fibrinolytic activity, most of the antifibrinolysin in the plasma is presumably excluded from the euglobulin precipitate. The euglobulin lysis time is thus probably an expression of total fibrinolytic activity without interference from antifibrinolysin, even though large amounts of the latter may be present in the native plasma from which the euglobulins are precipitated. Alterations in the lysis times of such a system will thus be due mainly to changes in one or more components of the fibrinolytic system itself.

Guest (1954) found an increase in antifibrinolysin during pregnancy. Our present findings confirm and extend this observation by showing that this excess antifibrinolysin is rapidly diminished within 24 hours of delivery. Our results thus disclose at least two contributory factors that may account for changes in fibrinolytic activity after delivery. These are, first, an increase in fibrinolysin (or precursors) and, secondly, a decrease in antifibrinolysin.

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THE POSSIBLE RELATIONSHIP BETWEEN THE ANTI-HEPARIN ACTIVITY OF SERUM AND THROMBOSIS

BY

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Stored serum reduces the anticoagulant effect of heparin on the clotting times of normal plasma. This is also well marked with sera from patients with Christmas (factor IX) deficiency, with factor VII-deficient sera, and in sera derived from patients treated with phenindione with a gross defect in thromboplastin generation. The possible relationship between antiheparin activity of serum and heparin resistance in recent thrombosis is discussed. The antiheparin agent resembles factor VII and Christmas factor in being present in excess in serum, adsorbed and subsequently eluted from alumina. Unlike these, however, it does not appear to be appreciably reduced by phenindione treatment. It appears to have some properties in common with those described for the thrombotic agent of serum described by Wessler and his colleagues. It may play a part in the increased coagulability associated with thrombosis from the release of serum products into the circulation, although its relationship to the production of thrombosis in man remains to be established.

The observation that heparin "resistance" is associated with active thrombosis has attracted a good deal of attention in recent years. The anticoagulant effect of heparin was seen to be less marked when it was given to patients with recent thrombosis than when it was given to other types of patient (Crafoord, 1937; de Takats, 1943). A heparin tolerance test *in vitro* was devised by Waugh and Ruddick (1944), and this was used by Ogura, Fetter, Blankenhorn, and Glueck (1946) to demonstrate that clotting took place more rapidly in cases of thrombosis than in controls. Silverman (1948) improved the test by the use of plasma to facilitate the reading of the end-point. I have used a modification of this test, the heparin plasma clotting test, which is performed under standardized conditions. With this technique it has been shown that in cases of vascular occlusion there is a significantly shortened clotting time (Poller, 1954, 1955, and 1956). A further study of this problem showed that plasma factor VII activity was increased as was thromboplastin generation associated with shortened heparin plasma clotting times (Poller, 1957) in a group of patients with recent thrombosis. It was subsequently observed that normal stored serum and serum preparations rich in factor VII activity reduced the effect of heparin on plasma clotting times (Poller, 1959).

The aim of the present investigation was to determine whether the antiheparin effect of serum was due to factor VII or to some other agent in serum. Serum was used in preference to products of alumina adsorption, as it was found that the activity of serum in neutralizing the anticoagulant action of heparin was quite marked, and it seemed preferable to use the more natural product. Serum may be a product of little physiological importance, but it may be released into the circulation following thrombus formation, and may be responsible for heparin "resistance."

To demonstrate the agent responsible for the antiheparin activity, observations were made on the effect of sera from patients with various coagulation defects on plasma clotting in the presence of heparin. Two sets of controls were used: (1) Normal saline to control the dilution effect, and (2) serum obtained from normal healthy adults. Sera with coagulation factor deficiencies were obtained from patients with Christmas disease (factor IX deficiency), from patients with pure factor VII deficiency, and from patients being treated with phenindione who showed a gross defect in thromboplastin generation.

Method

Reagents.—The following were used:

Calcium Chloride.—0.18%.

Normal Substrate Plasma.—Fresh oxalated plasma was obtained from 5 ml. venous blood by adding it to a mixture of potassium and ammonium oxalates.

(Stock solution (0.21 ml.) containing 1.6% potassium oxalate and 2.4% ammonium oxalate is put into a bijou bottle and left to dry overnight.) The blood and the oxalate anticoagulant were then thoroughly mixed after collection with the minimum of trauma and haemostasis, and the specimen spun at 1,500 r.p.m. in an M.S.E. angle centrifuge to obtain a platelet-rich plasma.

Heparin (Pularin (Evans) 10 units/ml.).—This solution was found to be stable for at least six months if kept at 4° C. when not in use, and a sterile technique employed to remove samples from the stock bottle.

Diluted Heparin.—The stock solution of heparin was diluted to a strength of 2 units/ml. immediately before use.

Test Sera.—Sera were obtained from healthy normal subjects and from patients with serum coagulation factor deficiencies from venous blood which was allowed to clot in glass test-tubes. The sera were separated by centrifuging, and the resulting serum removed and stored for 24 hours at 4° C.

Technique.—Four rows of three tubes (3 in. × ¼ in.) each were placed in a rack in a 37° C. water-bath, and allowed to warm. Normal plasma (0.2 ml.) and 0.1 ml. diluted heparin were added to each tube. To the first row of tubes increasing volumes of normal saline were added, 0.1 ml. to the first tube, 0.2 ml. to the second tube, and 0.3 ml. to the third tube. To the second row of tubes normal serum was added instead of saline, in the same increasing volumes. In the third and fourth rows of tubes two different test sera from different types of coagulation defect were compared by adding them, in the same increasing volumes, to their own row of tubes. The plasma clotting time was measured by adding 0.2 ml. of calcium chloride to each tube, mixing thoroughly, and tilting at 15-second intervals until a fibrin clot formed. The tubes of all four rows used in each experiment were recalcified at five-second intervals to eliminate the effects of storage *in vitro* on activating the coagulation mechanism. It was necessary to include a saline and normal serum control for each separate investigation, as the clotting times of normal plasma showed a good deal of variation in the presence of added heparin.

Results

The results are given in the Table. Those in the first row of tubes containing normal saline show that simple dilution with saline has no appreciable effect on the clotting time. The results in the second row of tubes containing normal serum show there is a significant shortening of the clotting time in the presence of heparin anticoagulant. All normal sera tested showed appreciable antiheparin activity.

TABLE
RESULTS

		0·1 ml.	0·2 ml.	0·3 ml.
Control 1. Normal saline (total 15)	Mean clotting time (min.)	7·3	6·9	7·1
	Standard deviation ..	2·26	1·98	2·0
Control 2. Normal serum (total 15)	Mean clotting time (min.)	3·2	2·9	2·5
	Standard deviation ..	1·8	1·56	1·34
Christmas serum (total 6)	Mean clotting time (min.)	4·0	3·7	3·6
	Standard deviation ..	0·5	0·3	0·59
Factor-VII-deficient serum (total 9)	Mean clotting time (min.)	3·6	2·6	2·5
	Standard deviation ..	1·56	1·41	1·33
Serum from phenindione-treated cases with deficient thromboplastin generation (total 7)	Mean clotting time (min.)	2·4	2·1	1·9
	Standard deviation ..	0·39	0·6	0·45

The effect of sera obtained from cases with severe Christmas disease in reducing the anticoagulant effect of heparin was well marked in all six cases, and, although rather less than in the group of parallel control normal sera, the difference was not significant. Thus Christmas factor does not appear to be the important antiheparin agent of serum.

Specimens were obtained from nine patients suffering from factor VII deficiency. Seven were patients treated with phenindione whose thromboplastin generation was 100% although prothrombin activity measured by Quick's method and the "P and P" method was depressed. Two patients were subjects with congenital factor VII deficiency. In this group of nine patients serum also significantly shortened the plasma clotting times in the presence of heparin.

A further group tested was a series of seven patients on phenindione whose thromboplastin generation was less than 1%. The thromboplastin defects during phenindione treatment result from the depression of Christmas factor and Prower-Stuart factor. There was no significant difference between this group and parallel normal control sera. Unlike factor VII and Christmas factor, Prower-Stuart factor is not activated during clotting (Johnson, Ferguson, O'Hanlon, and Black, 1959), and therefore was not likely on theoretical grounds to be the active antiheparin principle of serum.

As van Creveld and Paulssen (1951) have demonstrated that an antiheparin factor is present in platelets, it seemed important to eliminate the possibility that the effect may be due to the action of platelet extracts in serum. Blood was therefore collected into a siliconed syringe, transferred

to a siliconed tube, and the platelets spun out by centrifuging at 5,000 r.p.m. The platelet-deficient plasma was then removed and allowed to clot in glass, the resulting serum being tested the following day in the usual manner. The results of six sera obtained in this manner showed no significant difference from sera obtained from the same subjects collected in the usual manner. The antiheparin effect, therefore, does not seem to depend on factors released from the platelets during clotting.

The effect of adsorption by alumina was also observed. It has previously been shown that the antiheparin agent in serum could be adsorbed and subsequently eluted from alumina. Serum was obtained from subjects without coagulation defect. It was observed for its antiheparin effect before adsorption, and then 0.1 ml. of alumina was added to 1 ml. serum and the tube left in a water-bath for four minutes. The serum was then removed from the alumina by spinning in a centrifuge, and the adsorbed serum tested for antiheparin activity. In none of the adsorbed sera tested in this manner was there any evidence of antiheparin effect, and indeed in three the clotting time was prolonged, perhaps due to antithrombins remaining in adsorbed serum.

Discussion

The demonstration of the presence of a powerful heparin antagonist in serum appears of importance in view of the established association between heparin "resistance" and recent thrombosis. Heparin "resistance" may play a part in predisposing to thrombosis, but on the other hand it may in part at least be a secondary effect resulting from the release of serum products from the thrombus into the circulation. In support of this view is the observation that the shortest heparin clotting times appeared in cases in which large blood vessels were occluded. Patients with thrombophlebitis of large veins were found to have significantly shorter heparin plasma clotting times than those with myocardial infarction (Poller, 1959).

The antiheparin activity of serum may play some part in the production of intravascular clotting, even if this is only as an aggravation of a pre-existent clotting tendency. In the last few years several investigators have claimed that measurable amounts of heparin exist in the circulating blood (Freeman, Engelberg, and Dudley, 1954; Engelberg, 1958; Nillson and Wenckert, 1954; and others). Mast cells, the source of natural heparin, have been demonstrated in the walls of veins and arteries by Sundberg (1955) and Pomerance (1958). Both these authors reported an increase in the number of these cells in the vessels at the site of thrombus formation. The possibility that this may be a physiological response to the release of antiheparin serum products from the thrombus has been suggested (Poller, 1958).

The fact that antiheparin activity is not reduced by phenindione is somewhat surprising. When the heparin plasma clotting test was used as a method of control of treatment by dicoumarol and phenindione in a previous investigation, accelerated clotting, i.e., a clotting time shorter than the minimum normal, with further thrombotic episodes was found on many occasions at "therapeutic levels" of prothrombin activity (prothrombin times greater than twice the controls), and was regarded as an indication for additional heparin treatment. It is possible that such heparin resistance may have been due to the liberation of serum products into the circulation.

Wessler (1955) and Wessler, Reiner, Freiman, Reimer, and Lertzman (1959) have demonstrated the presence of a "thrombotic" agent in serum using a different technique. They have used experimental animals and produced thrombosis by the infusion of serum products. They have found that the serum product responsible could be adsorbed from serum, and obtained by subsequent elution as in the case of the antiheparin agent. This factor has also been found in factor-VII-rich solutions, and in later work they found that it was also present in the serum of patients on dicoumarol treatment. This led Wessler and his colleagues to the view that heparin may be preferable to dicoumarol as an anticoagulant drug (Wessler, Ballon, and Gilbert, 1956).

It appears from indirect evidence that the coagulant factor of serum demonstrated experimentally by Wessler may be related to the antiheparin agent of serum demonstrated by the author. The exact importance of such a serum product in the pathogenesis of thrombosis in man remains to be established, but there is some evidence to suggest that it may play a part in the production of the increased coagulability associated with recent thrombus formation.

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AMINOACIDURIA AND MEGALOBLASTIC ANAEMIA

BY

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Urinary amino-acid chromatograms from 23 patients with megaloblastic anaemia have been studied before and after therapy. The most consistent abnormality was an increased taurine or increased taurine/glycine ratio. This was not related directly to deficiency of vitamin B_{12} or folic acid or to the degree of anaemia.

The occurrence of aminoaciduria in patients with untreated pernicious anaemia was reported by Weaver and Neill (1954). Keeley and Politzer in 1956, using one-dimensional chromatography, confirmed its presence in two patients with pernicious anaemia. They found none in seven patients with megaloblastic anaemia of pregnancy and two infants with malnutrition and megaloblastic anaemia, so they therefore suggested that aminoaciduria was a further method for differentiating pernicious anaemia from the other megaloblastic anaemias. Crane, Hayes, and de Gruchy (1958) studied nine patients with pernicious anaemia and concluded that there was no characteristic pattern. Nevertheless, Weaver and Neill (1954) noted in particular a large increase in taurine concentration in five of their patients with pernicious anaemia and this finding was confirmed by Todd (1959) in the most extensive study to date on this subject. He showed that the mild aminoaciduria would occur in any of the megaloblastic anaemias in relapse and suggested that a pronounced increase of taurine was more common in pernicious anaemia and most likely to be found in the megaloblastic anaemias due to deficiency of vitamin B_{12}.

The purpose of this communication is to record our observations on the aminoaciduria in 23 patients with megaloblastic anaemia due to a deficiency of vitamin B_{12} or to a disturbance of the metabolism of the pteroylglutamates. Control observations were made in normal subjects and patients with hypochromic anaemia due to iron deficiency.

Clinical Material

Fifteen patients with megaloblastic anaemia and deficiency of vitamin B_{12} were studied. This group consisted of 10 patients with pernicious anaemia, having a macrocytic anaemia, megaloblastic erythropoiesis, serum level of vitamin B_{12} less than 105 $\mu\mu$g./ml. (Meynell, Cooke, Cox, and Gaddie, 1957), histamine-fast achlorhydria, normal excretion of faecal fat, and normal radiology of the stomach and small intestine together with an optimal haematological response to vitamin B_{12}. The remaining five patients had a megaloblastic anaemia in association with total gastrectomy, partial gastrectomy, adult coeliac disease, or regional enteritis (two patients). The eight patients with a megaloblastic anaemia but without evidence of deficiency of vitamin B_{12} included five patients with adult coeliac disease fulfilling criteria laid down elsewhere (Cooke, 1958), and three in association with pregnancy, two a week before and one a week after delivery and all remitting completely with folic acid. Control observations were provided by 10 normal subjects working in hospital without anaemia or evident disease and five patients with hypochromic iron deficiency anaemia, four due to chronic blood loss and one due to nutritional causes.

Methods

All subjects were on a normal hospital diet. Urine specimens were collected for two to three days before therapy and thereafter intermittently in the patients up to 15 days. Random observations on some of the patients were made subsequently up to eight months. Specimens were passed on rising, preserved with thymol and stored in the refrigerator until used, when they were run on two-dimensional paper chromatograms. A urine volume containing 250 μg. total nitrogen (determined by the Kjeldahl method) was applied to the paper (Whatman No. 1). A mixture of ascending and descending chromatography was performed as suggested by Block (1950), using a glass tank 15½ in. by 10½ in. by 9½ in. and paper chromatograms 13 in. by 11 in. This procedure required little laboratory space and no additional temperature control. The solvents and developing reagent were in accordance with Dent's method (1948), solvent (1) phenol saturated with water in the presence of ammonia and potassium cyanide, and solvent (2) lutidine 2: water 1 in the presence of diethylamine. The

TABLE

NUMBER OF CASES WITH STRONGER THAN NORMAL AMINO-ACID EXCRETION

	Total No. Cases	Amino-acids Usually Detected in Normal Urine							Amino-acids Not Usually Detected in Normal Urine				
		Glycine	Taurine	Taurine Relative to Glycine	Serine	Alanine	Glutamine + Glutamic Acid	Histidine	Lysine	Ethanolamine	Threonine	Traces of Valine, Leucine, Phenylalanine, Tyrosine	Cystine Qualitative Test
Pernicious anaemia	10	3	5	5	5	4	3	1	5	2	1	4	5
Other B_{12}-deficient megaloblastic anaemia ..	5	1	0	2	2	3	0	0	2	0	1	3	0
Non-B_{12}-deficient megaloblastic anaemia ..	5	0	3	5	1	1	2	0	1	1	0	3	2
Megaloblastic anaemia of pregnancy	3	2	0	0	1	2	1	1	0	0	0	2	1
Hypochromic anaemia	5	2	1	1	1	3	1	2	2	0	0	0	2

Methylhistidine and β-amino isobutyric acid excretion normal in all the above cases.

chromatograms were run in each solvent overnight, dried, sprayed with 0.1% ninhydrin in n-butanol, and the colours allowed to develop slowly overnight. The squares were then heated for a few minutes at 95–105° C.

By using this standard developing procedure the strength of the spots (numbered on an arbitrary colour scale 1–10) was compared visually with that obtained on the chromatograms of urines from normal subjects. Cystine was detected qualitatively in the urine by the cyanide-nitroprusside test. In some instances, this was confirmed by running oxidized chromatograms (Dent, 1948).

Results and Discussion

The results of 140 chromatograms are summarized in the Table.

In agreement with other workers, the most consistent abnormality in the urine amino-acids in Addisonian anaemia was an increased taurine excretion (five out of 10 patients). In five patients with non-Addisonian pernicious anaemia and a megaloblastic anaemia due to deficiency of vitamin B_{12}, none had an increased taurine excretion, but in two of them taurine excretion relative to glycine was increased. In normal controls taurine excretion was usually less and never more than that of glycine. This incidence in Addisonian anaemia is less than that reported by Crane *et al.* (1958), five out of seven patients, by Neill and Weaver (1958), 12 out of 16, and Todd (1959), 22 out of 24 patients. A possible explanation is that the urine specimens studied by us were obtained in a fasting state whereas pooled 24-hour specimens were used by other investigators. The daily diet providing an extra load may have accentuated the underlying defect.

The increased taurine excretion and the increased taurine-glycine ratio in the seven patients out of 15 with a megaloblastic anaemia due to B_{12} deficiency were not related to the degree of anaemia. Furthermore, the increase was not directly related to deficiency of vitamin B_{12} since it occurred in all five patients with adult coeliac disease who had no demonstrable deficiency and also in one of the five patients with hypochromic anaemia. It is unlikely that the increase is solely due to the disordered metabolism resulting from megaloblastic erythropoiesis since the taurine spot on the urine chromatogram of the three patients with megaloblastic anaemia of pregnancy was very faint. Todd (1959) did not detect any taurine in six of nine patients with this condition.

Other changes in the urinary amino-acids detected were relatively small and, in view of their inconsistency, are difficult to interpret and of doubtful significance. In all but one patient the abnormal urine amino-acids had returned or were returning to normal within five to 15 days of beginning specific therapy, whether this was vitamin B_{12}, folic acid, or iron. At present, urine amino-acid chromatography cannot offer any specific diagnostic aid in the differential diagnosis of anaemia.

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ANTIBODY OF BLOOD-GROUP SPECIFICITY IN SIMPLE ("COLD") HAEMOLYTIC ANAEMIAS

BY

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Two cases of "cold" (simple) haemolytic anaemia are described. The serological investigation showed conclusively that the sera of both patients contained an anti-I antibody and that their cells were I positive. The antibody found is thus a true auto-antibody. This is in keeping with the findings obtained previously in the "warm" (hyperimmune) type of haemolytic anaemia.

The acquired haemolytic anaemias are characterized by the presence of antibodies, and Dameshek (1951) suggested their differentiation into two types, the one with antibodies more active at blood heat ("warm") and the other at lower temperatures ("cold"). One of us (Weiner, 1957a) designated them later as hyperimmune ("warm") and simple ("cold") haemolytic anaemias respectively.

An antibody may be defined as a plasma protein which reacts specifically with its homologous antigen. The blood-group specificity of an antibody can readily be recognized if cells are easily available which are not acted upon by this antibody, but difficult if such cells are scarce. In 1953 (Weiner, Battey, Cleghorn, Marson, and Meynell), it was shown for the first time that the antibodies which occur in "warm" (hyperimmune) haemolytic anaemias may have distinct and definable blood-group specificity. The antibody found in "cold" (simple) haemolytic anaemia was, however, still regarded as "unspecific." Crookston, Dacie, and Rossi (1956) investigated these antibodies and found a marked difference in their reactivity with different cells. Wiener, Unger, Cohen, and Feldman (1956), however, described a case of "cold" (simple) haemolytic anaemia in which the antibody had distinct blood-group specificity. They were able to find amongst many thousands of donors five whose cells did not react with the patient's antibody. They called the antibody anti-I, corresponding to the antigen I on the reacting cells. The non-reacting cells were supposed to lack the I property or to carry the property i.

The great scarcity of i cells prevented further investigation of other cases of "cold" (simple) haemolytic anaemia. Race and Sanger (1958) had in the meantime described a serum, Steg, which seemed to be identical with the serum described by Wiener *et al.* (1956). Later still we learned of the work done by Jenkins, Marsh, Noades, Tippett, Sanger, and Race (1960) and, through the generosity of Drs. Jenkins, Race, and Sanger, were supplied with two samples of i cells and also with anti-I serum of the "natural" type. This material made it possible to investigate the two patients with "cold" (simple) haemolytic anaemia who are the subject of this report.

Case Histories

Mrs. A., then 27 years of age, developed acute haemolytic anaemia 48 hours after the birth of her first child in September, 1956. A week later, her haemoglobin had fallen to 5.9 g.% (40%) with 15% reticulocytes. The liver and spleen were slightly enlarged. The serum bilirubin level was 1.4 mg.%. The haemolysis was controlled by 60 mg. prednisolone daily. Subsequently she remained well, though, whenever steroid treatment was stopped, the haemoglobin fell and the reticulocyte count increased. A daily maintenance dose of 5 mg. prednisolone controlled this. She had a relapse in February, 1959, after a feverish illness due to a urinary infection. Increased dosage of prednisolone brought the haemolysis again under control.

Mr. F. L., aged 77, was admitted to hospital with chronic bronchitis and emphysema, and in the course of a routine blood count in March, 1958, strong auto-agglutination of the red cells was noted. Haemoglobin was 11.8 g.% (80%) with a reticulocyte count of 3%, and the direct Coombs test was positive. He was

again admitted in November, 1958, with herpes zoster. Then he had a haemoglobin of 8.9 g.% (60%), W.B.C. 14,000 with a normal differential count, and reticulocytes 2.7%. Again the direct Coombs test was positive at room temperature and at 37° C. "Cold" agglutinins were reported to be present. An L.E. test and a Schumms test were negative. Haptoglobins were absent. The Wassermann reaction and the Donath-Landsteiner test were negative.

Material and Methods

Venous blood was delivered into six universal containers. Three of these contained acid citrate dextrose which was used in the proportion of 1 part to 9 parts of blood. One container with acid citrate dextrose and one without were immediately dropped into a "thermos" flask filled with water at 37° C., and another pair put into crushed ice; the third pair was kept at room temperature. All the specimens were handled subsequently at the temperatures into which they were first delivered. The cells of the specimens taken into acid citrate dextrose were washed three times with large amounts of saline of the appropriate temperature and eluates were prepared from them, both by the method described previously (Weiner, 1957b) and the heat method (Landsteiner and Miller, 1925). The sera were stored at −25° C. until used. The cells and sera thus collected will be referred to as "37° C.," "22° C.," and "4° C." cells and sera. The i cells were those described by Jenkins *et al.* (1960) (donor M.) and Tippett, Noades, Sanger, Race, Sausais, Holman, and Buttimer (1960) (donor R.). The i cells had been kept frozen in glycerol, and to make a valid comparison cells which had been similarly kept were used as controls. Whenever possible the patient's own cells were included among the test cells. When the alcohol method (Weiner, 1957b) was used the eluates were taken into AB serum and with the heat method into saline. Cells were made up as 2% saline suspensions. Both Löw's (1955) papain method and papainized cells were used. The latter were prepared using Löw's activated papain diluted with 19 parts of the buffer solution. This diluted enzyme was used in equal parts with packed red cells. The mixture was incubated for 15 minutes in a water-bath, washed once, and again a 2% suspension was prepared. When Löw's method was used, 1 part of serum or eluate was mixed with 1 part of the papain solution. When the patient's own cells were used as test cells, they were used unmodified.

All tests were read "blind"; the material was coded by an assistant and the code disclosed only after the results had been read.

Results

Mrs. A. was found to be Group A_1 rhesus positive, phenotype CCDee. The specimens were obtained at the beginning of March, 1959, when she was in hospital having suffered the relapse described above. Serum was also available which had been collected on her first admission to hospital in 1956 and gave identical results. The direct Coombs test on Mrs. A.'s cells gave a distinctly positive result which varied from + to ++. Auto-agglutination was marked in the 4° C. specimen, but the agglutinates could be broken up by shaking in saline. Very small agglutinates were left, but Coombs serum enhanced this agglutination very strongly. No auto-agglutination was seen in the 37° C. specimen, but the positive Coombs test indicated that the cells, even at this temperature, were coated, though slightly.

Mr. L. was Group A_1 rhesus positive, phenotype ccDE. (As the three samples of anti-e available were all of the incomplete variety, it was not possible to establish with certainty whether the patient's cells carried the e antigen.) The result of the Coombs tests was similar to that described above. Auto-agglutination was fairly strong in the 4° C. specimen but was again greatly enhanced by Coombs serum.

The investigation of the sera obtained from the sample from Mrs. A. gave a somewhat intriguing result. When titrated, the three sera which were collected into different temperatures scored differently.

As Table I shows, the highest score was obtained with the 37° C. serum, which seems to imply that at a lower temperature the cells of the patient absorbed some of the antibody. The serum of Mrs. A. was then put up against a random cell from our cell panel, i cells, and her own cells obtained from all her three specimens. Serum M., which contains a "naturally" occurring anti-I, was put up in parallel together with a Group AB serum as a control. The results are shown in Table II.

From this it can be seen that i cells were not agglutinated by any of the sera used but that both the patient's own sera and serum M. agglutinated her cells very strongly indeed. A similar result was obtained when the sera from Mr. L. were investigated (Table III).

TABLE I

TITRATION OF MRS. A.'S THREE SERA AGAINST ONE RANDOM UNMODIFIED CELL AT 4° C.

Sera Separated at									Scores
4° C.	4	4	2	W	—	—	—	—	34
22° C.	4	4	3	W	W	—	—	—	38
37° C.	4	4	3	3	1	W	W	—	53

4=++++ 3=+++ 2=++ 1=+ W=weak — = neg.
Scores: 4=12 3=10 2=8 1=5 (1)=3 W=2.

TABLE II

	Sera				
	Donor M.	Mrs. A. Serum Separated at:			AB
		4° C.	22° C.	37° C.	
Control cell ..	4	4	4	4	—
Cells donor M. ..	—	—	—	—	—
4° C. } Cells	4	2	3	4	—
22° C. } Mrs. A.	4	3	4	4	—
37° C. }	4	3	4	4	—

Incubated at 4° C.
4=++++ 3=+++ 2=++ —=neg.

TABLE III

	Sera				
	Donor M.	Mr. L. Sera Separated at:			AB
		4° C.	22° C.	37° C.	
Random group A cells ..	4	3	3	3	—
Cells Mr. L. separated at 37° C.	4	3	3	3	—
Cells donor M.	—	—	—	—	—

Incubated at 4° C.
4=++++ 3=+++.

Again i cells (donor M.) were not agglutinated, and the patient's sera and serum M. agglutinated strongly the patient's cells. Tests using cells from donor R. gave identical results.

Jenkins *et al.* (1960) demonstrated that their anti-I serum acted on cord blood cells much less strongly than on cells from adults. We obtained identical results with the sera from our two patients, and Table IV gives the scores obtained when the two 37° C. sera were titrated against cells from adults chosen at random and three cord blood samples.

Jenkins *et al.* (1960) have shown that the i cells of donor M. contain very little H substance. Tippett *et al.* (1960) confirmed this in their donor R., but obtained much higher scores with i cells from three other donors. It is thus obvious that an anti-H antibody might give reactions similar to anti-I with certain i cells. The following experiments showed that the sera of our patients did not contain anti-H but anti-I. Saliva from secretors did not diminish the titre of the antibody, confirming the findings of Jenkins *et al.* (1960). Further, Drs. Race and Sanger were kind enough to test the sera of our patients against a sample of "Bombay" blood which does not contain any H substance. The cells were strongly agglutinated. If our sera had been anti-H, one would also have expected that Group O and Group A_2 cells would have reacted more strongly than A_1 cells. This, however, was not the case.

Both our patients were Group A_1, the same, therefore, as i cells at our disposal. Their cells were, as shown above, strongly agglutinated by the "naturally" occurring anti-I and by the sera from both patients. We titrated random Group O, A_1, A_2, the two i cells, and the 37° C. cells from our patient L. against *Ulex Europaeus*.

Table V shows the result of this titration. Group O and Group A_2 scored highest, Group A_1 less, and the i cells lowest. Mr. L.'s cells gave a score of 23 showing that they contained the amount of H substance expected of A_1 cells.

The eluates were investigated in a similar way. (No eluate could be prepared from cells taken into room temperature from Mr. L., as unfortunately the container broke in the centrifuge and it was not thought essential to obtain a further sample.) The eluates prepared by the alcohol method reacted more strongly.

It can be seen from Table VI that the eluates also showed blood-group specificity. The i cells were not agglutinated at all, in contrast to the patient's own cells. Eluates prepared from the 37° C. specimen of Mrs. A. did not produce any agglutination when used with unmodified cells. Those prepared from a similar specimen obtained from Mr. L. acted quite well.

Wiener *et al.* (1956) mentioned that ficinized i cells were agglutinated by the serum of

TABLE IV

SCORES OBTAINED AFTER TITRATING MRS. A. AND MR. L. 37° C. SERA AGAINST CELLS FROM THREE RANDOM ADULT DONORS AND FROM THREE RANDOM CORD BLOOD SAMPLES FROM FULL-TERM BABIES

Sample No.	At 22° C.		At 4° C.	
	Mrs. A.	Mr. L.	Mrs. A.	Mr. L.
9735	35	37	90	117
9736	36	51	95	121
9737	61	93	109	132
Baby 1	2	—	39	25
,, 2	5	—	51	14
,, 3	2	—	42	9

TABLE V

TITRATION OF CELLS WITH *ULEX EUROPAEUS* EXTRACT

											Scores
Gp. O cell ..	4	4	4	4	2	W	—	—	—	—	58
37° C. Mr. L.	2	1	1	(1)	W	—	—	—	—	—	23
Donor R. ..	W	W	—	—	—	—	—	—	—	—	4
,, M. ..	W	W	—	—	—	—	—	—	—	—	4
Gp. A_1 cell	2	2	1	W	W	—	—	—	—	—	25
Gp. A_2 ,,	4	4	4	4	3	2	W	—	—	—	68

Incubated at 4° C.
4=++++ 3=+++ 2=++ 1=+ W=weak.
Scores: 4=12 3=10 2=8 1=5 (1)=3 W=2

TABLE VI

ELUATES MRS. A.

	Alcohol Eluate			Heat Eluate		
	4° C.	22° C.	37° C.	4° C.	22° C.	37° C.
Random A_1 cells	4	3	—	3	3	—
Cells donor M.	—	—	—	—	—	—
4° C. Cells	2	1	—	1	1	—
22° C. Cells	3	1	—	1	1	—
37° C. Mrs. A.	3	2	—	2	1	—

ELUATES MR. L. (ALCOHOL ELUATES)

	4° C.	37° C.
Random A_1 cells	4	3
Cells donor M.	—	—
37° C. cell Mr. L.	3	2

4=++++ 3=+++ 2=++.
Scores: 4=12 3=10 2=8.

their patients. We used papain to modify the cells. The activity of our patients' sera was much enhanced by papain both when this enzyme was used to modify the test cells or on its addition to the sera. They behaved in the way described by Wiener *et al.* (1956) and confirmed by Jenkins *et al.* (1960). They also agglutinated i cells, though to a much lower titre. The agglutination was further enhanced when the tests were incubated at low temperatures. The eluates behaved differently; although their activity was also much enhanced when papain was used, i cells were only weakly or not agglutinated at all.

It was of interest to note that the 37° C. eluate of Mrs. A.'s specimen which had shown no activity against unmodified I cells could be made to react when papain was used and when the tests were put up at 4° C. This shows that even at 37° C. the cells of our patients were coated by antibody, though more sensitive methods had to be used to show its presence in the eluate. As Table VII shows, the eluates both from Mrs. A. and Mr. L. agglutinated the 37° C. cells, those of Mr. L. as strongly as the control cells.

Discussion

Both patients suffered from typical "cold" (simple) haemolytic anaemia. All the symptoms and signs commonly seen were present in both patients, though Mr. L. suffered mainly from emphysema and bronchitis and was found to be anaemic on one occasion only.

The sera of both patients contained a powerful antibody which acted best at a low temperature. The properties of the sera were identical with those described by Wiener *et al.* (1956) and Jenkins *et al.* (1960). The antibody was not anti-H, as it reacted with "Bombay" blood and was not inhibited by secretor saliva. The cells obtained from the specimens kept at 4° C. did not react as well as the cells obtained from specimens collected into 22° C. and 37° C. (see, for instance, Table II). The sera behaved in the opposite way. Those obtained at a higher temperature gave a better titre than sera from the specimen at 4° C. The fact that the cells of both patients were agglutinated by two "naturally" occurring anti-I sera and were also agglutinated by the immune anti-I of their own sera, the sera of the other patient, and the eluates indicates clearly that they are I. Further proof was obtained by the fact that a powerful antibody could be eluted which again had anti-I specificity. This antibody could only have been found on the cells if they contained the homologous antigen, as we were not able to elute any antibody from the cells of donor M. or R. In addition, the cells had a normal complement of the H antigen which, as Jenkins *et al.* (1960) have shown, is low in some i cells. Both our patients were therefore I positive.

There is a definite difference in the behaviour of the "naturally" occurring anti-I and the anti-I in haemolytic anaemia. When i cells were enzyme treated, the sera of our patients agglutinated them to a certain extent though certainly not to as high a titre as I cells. Whereas a low temperature was necessary to bring about a weak agglutination of enzyme-treated cells from donor R. by serum

TABLE VII

REACTIVITY OF PAPAINIZED CELLS WHEN TESTED WITH SERA AND ELUATES OF MRS. A. AND MR. L.

	Mrs. A.						Mr. L.					
	Sera			Eluates			Sera			Eluates		Donor M. Serum
	37° C.	22° C.	4° C.	37° C.	22° C.	4° C.	37° C.	22° C.	4° C.	37° C.	4° C.	
Random A_1 cells ..	4	4	4	2	3	4	4	4	4	4	4	3
Cells from donor R. ..	4	4	4	—	W	W	4	4	4	—	1	—
37° C. cells Mr. L. ..	4	4	4	3	4	4	4	3	2	3	4	4

Incubated at 4° C. 4=++++ 3=+++ 2=++ 1=+ W=weak.

T

M., serum R. did not agglutinate cells from donor M. under any conditions (Tippett *et al.*, 1960). (It is interesting to note that the eluates behaved very similarly.) This difference between a "naturally" occurring antibody and an immune antibody is not unique and is seen on occasions in other fields. It is known that when an animal is immunized against an antigen the specificity of the antibody, at first narrow, subsequently widens to include related antigens (Raffel, 1953; Boyd, 1956).

Our results do not help to explain the different serological behaviour of antibodies of the "natural" or "immune" variety. Everyone is exposed to the same antigenic stimuli, but only those who lack the homologous antigen will develop the corresponding antibody, that is, as long as the response to this antigen is "normal." In abnormal conditions, antibodies against antigens carried on the patient's cells may develop.

We cannot agree with the point of view of Wiener *et al.* that i people are liable to develop haemolytic anaemia due to their forming an anti-I. In the first instance, we have shown that both our patients were I positive. Wiener *et al.* have not produced any proof at all that their patient was really i. The poor reaction of her cells to her own serum could be explained by their being heavily coated with her own antibody. Wiener *et al.* rejected this possibility without apparently carrying out further investigations. If an elution was tried, the result was not published. From the red cells of both our cases, we could elute with ease a powerful antibody. Even the less sensitive heat method yielded a good eluate. Furthermore, it is difficult to imagine that an antibody formed by a patient who is lacking the homologous antigen should be the cause of a severe illness. It is hard to understand their conclusion that: "If further studies show that other similar cases of acquired haemolytic anaemia *due to cold auto-antibodies* (our italics) behave similarly this could provide a partial explanation for the rareness of this condition since presumably only I-negative individuals, who are quite rare, would be apt to produce such antibodies." "Cold" (simple) haemolytic anaemias are by no means as rare as i people. In a short time, we have seen quite a number of "cold" (simple) haemolytic anaemias, but we have not yet managed to find an i cell amongst the thousands of specimens which pass through our hands. Antibodies produced by people against an antigen which they themselves lack are in fact harmless as, e.g., anti-rhesus antibodies in rhesus-negative people. Similarly one would not expect I-negative people, who develop anti-I, to show any signs of illness, and in fact, as Jenkins *et al.* have shown, the presence of anti-I in a truly i patient is compatible with full health.

These results further support the assumption of the blood-group specificity of the antibodies in acquired haemolytic anaemias. Even if our present methods and the availability of cells do not allow us as yet to define this specificity in every case, their behaviour is such that specificity is much more likely than an "unspecific" antibody. Crookston *et al.* (1956) explain that by the term "non-specific antibody" they mean that "the receptor for the particular antibody is presumed to be present on the surface of all human red cells." Until really all human cells, an obviously impossible undertaking, have been tested, this assumption is not tenable and liable to lead to wrong conclusions, as the present investigation has shown. Wiener *et al.* (1956) by not assuming "non-specificity" were able to provide donors for their gravely ill patient which would not have been possible had they acted according to the assumption that "all cells" carried the antigen. Up till now, quite a number of I-negative people (or nearly I-negative) have been found (Tippett *et al.*, 1960). Their cells will help to increase our knowledge of blood-group systems in man, but, more important still, they are potential donors for patients with acquired haemolytic anaemia who require transfusion.

This work could not have been done at all had it not been for the help of Drs. Race and Sanger and the cells and sera supplied both by them and by Dr. Jenkins and his colleagues. We should like to express our most sincere thanks to them.

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SYPHILITIC PAROXYSMAL COLD HAEMOGLOBINURIA

CASE REPORT AND STUDY OF THE COOMBS TEST

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A case of congenital syphilis which presented with paroxysmal cold haemoglobinuria is described.

The patient had aortic incompetence, and the significance of this finding is discussed.

The Coombs test was studied, and positive results appear to be due to interaction between antiglobulin serum and complement adsorbed on to the test cells, this adsorption being promoted by the cold antibodies.

Syphilitic paroxysmal cold haemoglobinuria is an uncommon condition, and, unlike many of the other haemolytic diseases, the serological mechanisms involved are by no means clear. Thus, according to Darmady and Davenport (1954), the Coombs test is negative, although Jordan, Pillemer, and Dingle (1951) had stated that in the presence of an excess of complement the indirect Coombs test is positive. Dacie (1954) thought that in this condition the optimum concentration of antiglobulin serum was considerably greater than that needed for routine blood grouping, and some batches would therefore give false negative results.

Dacie, Crookston, and Christenson (1957) studied a group of patients with cold incomplete antibodies, and considered that a positive Coombs test was due to interaction between the antiglobulin serum and the cold antibody. They studied one patient with cold haemoglobinuria and Donath-Landsteiner antibodies, and found that he behaved similarly.

We report here a patient with congenital syphilis, who presented with paroxysmal cold haemoglobinuria and in whom detailed studies of the Coombs test were made.

Case Report

Private T. C., aged 19 years, was admitted to Catterick Military Hospital in April, 1957, because he had been passing red urine.

He had been perfectly well until the age of 14 when, after playing in the snow for two or three hours, he felt generally unwell, hot and shivery, and when he passed urine he saw that it was dark red. There was no frequency or dysuria, no loin pain, and he did not think that he was jaundiced. Over the next 36 hours the urine gradually cleared, but he was admitted to the local hospital for investigation. Urine examination showed no abnormality, and, although an intravenous pyelogram had failed to outline the right upper calyces, retrograde pyelography showed no abnormality. Cystoscopy was normal.

Several weeks later, again following exposure to cold, when he had been shovelling snow, he passed red urine for about 12 hours. When aged 15 he left school and started work in a railway workshop. While so employed he suffered three further attacks in which he passed red urine, each one following exposure to cold. The patient had been so sure of this relationship that he had changed his job for one in the warmer surroundings of a printing works.

In March, 1957, he was called up for National Service and passed the routine medical examinations satisfactorily. One week later, however, following a three-hour spell of fatigues during which time he was peeling potatoes, and his hands were immersed in cold water, he again felt unwell, shivered, and passed red urine. He was admitted to a surgical ward, but no abnormality was found on clinical examination; as the urine did not contain red cells no further investigations were done and he was returned to his unit. Two days before his readmission he had again been working in the open air on a cold day and had thereafter passed dark urine.

There was no other relevant history and, in particular, there was no illness resembling acute rheumatism. His parents, aged 42, were both well, as were his 16-year-old brother and 14-year-old sister. His mother had had no miscarriages or stillbirths.

Examination showed a fit-looking boy, of below-average intelligence and of average physique. The

upper incisor teeth were peg-shaped, but the molars were normal. Other relevant findings were as follows:

Cardiovascular System. — Blood pressure was 145/55 mm. Hg. No cardiac enlargement was found, but a loud diminuendo diastolic murmur was heard down the left sternal border. A chest film and screening were normal, as was an electrocardiogram.

Central Nervous System.—The pupils were equal and regular, reacting well on convergence but very sluggishly to light. Ophthalmoscopic examination showed signs of old choroido-retinitis. Reflexes were normal.

Laboratory Investigations.—In order to carry out any test involving serum, it was necessary to allow separation to take place at 37° C. to prevent haemolysis.

Haematological Investigation.—Haemoglobin was 94% (13.9 g./100 ml.), white cells 7,600/c.mm. (differential count normal), red cells normochromic and normocytic, reticulocytes 0.6%.

Urine.—On admission the urine was dark brown and free from red cells, but spectroscopy showed strong absorption bands of oxyhaemoglobin. Twenty-four hours after admission the urine was normal.

Serum bilirubin was less than 0.5 mg./100 ml.

Schumm's test for methaemalbumin was positive.

Ham's test was negative.

Cold agglutinin titre was 1 in 8.

Serum protein electrophoresis was normal.

The Wassermann reaction was positive 1 in 320, and Kahn and Price precipitation reactions were positive 320 u.

A *Treponema pallidum* immobilization test was positive. The cerebrospinal fluid contained 4 lymphocytes/c.mm., protein 110 mg./100 ml. The Lange curve was 1555210000. The Wassermann reaction was positive 1 in 80.

Cooling the limbs by immersion in ice-cold water for 20 minutes was followed by the appearance, 30 minutes later, of haemoglobin in the urine. This had disappeared by three hours. The serum bilirubin level rose to 5.3 mg./100 ml. two hours after cooling, but had returned to normal levels within 24 hours. The patient was symptom-free throughout, and there was no change in the peripheral blood count.

The results of studies on the Donath-Landsteiner and Coombs tests, which were positive throughout, are described below.

Investigation of Patient's Family.—Blood taken from the patient's mother gave a positive Wassermann reaction, but that from the two younger children was negative. The father refused to have the test performed.

Treatment.—The patient was given bismuth, 0.2 g. intramuscularly, twice weekly, and a mixture of potassium iodide, gr. 10, and liquor hydrarg. perchlor. B.P., 1 drachm three times daily, for two weeks. Subsequently procaine penicillin, 1 mega unit, was given intramuscularly daily for three weeks. The patient remained perfectly well throughout the course of treatment, and indeed throughout his entire stay in hospital.

After treatment he was boarded out of the Army and returned home. During the following year he remained symptom-free, with no recurrence of the haemoglobinuria.

Special Tests

Donath-Landsteiner Reaction.—Results obtained with serum from this case confirmed the results of other workers. The methods used were those described by Darmady and Davenport (1954).

(1) The maximum titre observed at any time was 1 in 8 serum dilution. (2) Negative results were consistently observed unless complement was present in the cold phase as well as in the warm phase. (3) Although positive results were obtained when sensitization was carried out at 4° C., no haemolysis was observed if sensitization was carried out at 22° C. and 37° C.

The Coombs Test.—Patient's serum was used fresh after separation at 37° C. or subsequently heated for half an hour at 56° C.

Normal fresh human serum was used as complement absorbed three times with fresh Group O cells at 4° C. for one hour.

Guinea-pig complement was pooled, normal, fresh serum absorbed three times with fresh Group O cells at 4° C.

Three batches of rabbit antihuman globulin serum from different sources were employed. These had all proved equally satisfactory in the identification of rhesus antibodies.

Anti-guinea-pig globulin serum was inactivated at 56° C. for half an hour, diluted, and absorbed three times with Group O cells at 4° C.

For test cells, fresh Group O cells, washed and made up to a 2% suspension, were used.

Human γ globulin was the preparation supplied for prophylactic immunization by the Lister Institute.

The methods described by Dacie (1954) and Dacie, Crookston, and Christenson (1957) were used.

Results

Effect of Different Batches of Antiglobulin Serum.—Three different batches of antiglobulin serum, A, B, and C, were used. The sera were first used at a dilution which was optimal for Rhesus testing; with antiglobulin serum A the direct Coombs test on the patient's cells was negative, with serum B it was doubtful, and with serum C unequivocally positive. The antiglobulin sera were then used at a higher concentration (a dilution which was just sufficient to ensure that unsensitized cells were not agglutin-

ated). Sera A and B now gave an unequivocally positive direct Coombs test, and the positive test previously obtained with serum C became stronger (judged by a more rapid agglutination and the appearance of larger cell aggregates). Similar results were obtained in the indirect Coombs test. With test cells sensitized in the cold by undiluted patient's serum, a positive Coombs test was obtained with all three antiglobulin sera, provided that they were used in the more concentrated form, the strongest result being obtained with serum C. When the test cells were sensitized with the patient's serum diluted 1/16, only antiglobulin serum C gave a positive result.

Effect of Variation in Temperature During Sensitization. — Using fresh normal patient's serum, without added complement, it was shown that a positive indirect Coombs test could best be demonstrated if the sensitization was performed at 0° C., was just present at 12° C., but was negative if sensitized at 20° C. and 27° C.

Need for Complement.—When heated patient's serum was used alone in an attempt to sensitize the cells, the Coombs test was negative. However, if doubling dilutions of the heated patient's serum were carried out in fresh human serum, which provided complement, the Coombs test was positive to a titre of 1/16. Adequate amounts of complement were present in the fresh unheated patient's serum because this same titre was obtained when test cells were sensitized with dilutions of unheated serum in saline, and it was not enhanced by the addition of normal serum as a further source of complement.

Effect of γ Globulin Added to Antiglobulin Serum.—The effect of adding increasing dilutions of human γ globulin to the antiglobulin serum used in the direct Coombs test on the patient's cells was studied and compared with the effect on the indirect Coombs test carried out on a Rhesus-sensitized system. The direct Coombs test was inhibited on the patient's cells only when a large amount of γ globulin was added, whereas with Rhesus-sensitized cells the Coombs test was inhibited by very small amounts of γ globulin. In the case of the direct Coombs test on the patient's cells, no inhibition occurred until the final dilution of the 10% γ globulin, added to the antiglobulin serum, was less than 1/32, whereas with Rhesus-sensitized cells inhibition was complete with dilution up to 1/2,000.

Indirect Coombs Test Sensitizing with Alternative Sources of Complement.—Test cells were sensitized by doubling dilutions of the patient's heated serum carried out first in fresh human serum and then in fresh guinea-pig serum as alternative sources of complement. The Coombs test was carried out in the normal way, using antihuman globulin serum, and also by substituting for the antihuman serum a rabbit serum prepared against guinea-pig serum (anti-guinea-pig globulin serum). The results are set out in the Table. When human complement was used during sensitization, there was strong agglutination with antihuman serum and a weaker reaction with anti-guinea-pig serum. When guinea-pig complement was used, the strength of the reaction was reversed, the strong reaction occurring with the anti-guinea-pig serum.

TABLE

AGGLUTINATION BY ANTIGLOBULIN SERA OF RED CELLS SENSITIZED BY DILUTIONS OF PATIENT'S HEATED SERUM CARRIED OUT WITH HUMAN OR GUINEA-PIG SERUM AS COMPLEMENT

Antiglobulin Serum	Serum Added to Anti-globulin Serum	Serum Used as Source of Comple-ment	Dilution of Patient's Heated Serum Used for Sensitization				
			½	¼	⅛	1/16	1/32
Antihuman ..	None	Human	++ ++	++ ++	++ +	+	−
Anti-guinea-pig	,,	,,	++	+	−	−	−
Antihuman ..	Guinea-pig	,,	++ ++	++ ++	++	+	−
Anti-guinea-pig	Human	,,	−	−	−	−	−
Antihuman ..	None	Guinea-pig	++	+	−	−	−
Anti-guinea-pig	,,	,,	++ ++	++ ++	++	+	−
Antihuman ..	Guinea-pig	,,	−	−	−	−	−
Anti-guinea-pig	Human	,,	++ +	++ +	+	−	−

The tests were then repeated using antihuman globulin serum to which guinea-pig serum had been added, and anti-guinea-pig globulin serum to which human serum had been added. The effect was to abolish the weaker reactions altogether, while leaving the strong reaction unchanged. It was concluded that the weak reactions were presumably due to cross-reactions between the antiglobulin serum and heterologous serum components.

Discussion

From the clinical viewpoint, this case presents two interesting features. The first of these relates to the patient's previous history, for it will be recalled that he was investigated on two occasions for "haematuria." This is a common story in such patients, and serves to emphasize the importance of examining the urine for pigments as well as red cells before discounting the patient's story. The second, and more important, feature was the presence of a diminuendo diastolic bruit

and wide pulse pressure. It seems possible that this patient had syphilitic aortic incompetence, as there was no evidence of previous acute rheumatism, yet there was clear evidence that he had congenital syphilis, and that his teeth, central nervous system, retinae, and antigen-antibody mechanisms were affected. There is no conclusive recorded evidence that such patients develop aortitis, but Bowie and Simpson (1953) described a 38-year-old male congenital syphilitic with aortic incompetence. Although their patient had had rheumatic fever at the age of 19, an aortic aneurysm was present, and the evidence was strongly in favour of a diagnosis of syphilitic aortitis.

The serological studies showed that our patient's Donath-Landsteiner antibody behaved in the Coombs test in a similar manner to the cold antibodies occurring in other conditions (Dacie *et al.*, 1954). Thus the positive Coombs test was due to the interaction of the antiglobulin serum with complement adsorbed on to the test cells. The Donath-Landsteiner antibody did not appear to interact with the antiglobulin serum but exerted its effect by causing complement to be adsorbed on to the cells.

This concept is consistent with some of the other findings: the importance of using antiglobulin serum at a higher concentration than the usual optimum for Rhesus testing; the variability of the results of the Coombs test with different batches of serum, and the persistence of positive results after the addition of γ globulin to the antiglobulin serum. These apparently anomalous results accord well with the idea that it is some additional antibody, other than the specific antiglobulin antibody, that is responsible for the positive results observed.

We are grateful to Professor J. V. Dacie, Lieutenant-Colonel C. E. Stewart, and Dr. P. N. Coleman for advice and encouragement; to Dr. J. H. Crookston for the anti-guinea-pig serum; to Dr. I. N. Orpwood Price for carrying out the T.P.I. test; and to Dr. C. V. Fitzgerald, of Hucknall, Notts, for following-up the patient's family.

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SERUM IRON DETERMINATION

BY

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A simple, rapid, and sensitive method for the determination of iron in serum or plasma is described.

The procedure is carried out at room temperature with 2 ml. of serum or plasma, or with 1 ml. if high values are expected; it can be applied to turbid or jaundiced samples, whether previously frozen or not.

An ethanolic solution of 4:7-diphenyl-1:10-phenanthroline is used to produce a coloured iron complex, the optical density of which can be measured in any suitable photometer, using either 10 or 20 mm. fused glass cuvettes or matched tubes of 1.1 cm. internal diameter.

Methods for the determination of serum or plasma iron have been reviewed by Heilmeyer and Plötner (1937), Hemmeler (1951), Laurell (1952), and Ramsay (1958). Wet ashing procedures have for many years given the most dependable results, but the labour involved in their performance is considerable. Most of the methods in routine use are based on Barkan's (1927) observation that adding acid to plasma liberates iron from the protein with which it is normally bound; the proteins are then precipitated, either by heat or by the addition of trichloracetic acid, and the iron is determined colorimetrically after the addition of some substance which will form a coloured complex with the free iron.

Dissociation of the plasma iron-protein complex (transferrin) has usually been effected by adding hydrochloric acid, the final concentration of which has varied from 0.1 N to 2 N.

A proportion of the liberated iron may be lost by adsorption on to insoluble protein during the precipitation process, especially if the preparation has been heated. This was demonstrated by Hill in 1930. He showed that it could be prevented by adding a reducing agent and by maintaining the *p*H at about 4 with an acetate buffer. His observations were confirmed by Fowweather (1934) and by Tompsett (1934) using different techniques, but were then lost sight of for some years.

Laurell (1952) pointed out that the optimal *p*H for achieving the rapid conversion of ferric to ferrous ions varies with the reducing agent used, and that, in the case of hydrazine sulphate, thioglycollic acid, and sodium dithionite, a *p*H of 4.5 to 4.9 is most suitable. This can most simply be maintained by buffering with a saturated solution of sodum acetate (Tompsett and McAllister, 1949).

Utilizing these principles, Ramsay (1953) obtained results which were 30–60 μg./100 ml. higher than those obtained from the same specimens when using several of the older methods. This was confirmed by Peters, Giovanniello, Apt, and Ross (1956), who also showed that in the presence of a reducing agent dilute hydrochloric acid gave complete iron recovery from specimens which had previously been frozen.

Thiocyanate, $\alpha\alpha'$-dipyridyl, and 1:10-phenanthroline have chiefly been used to form coloured complexes with the extracted iron. As the molar extinction coefficients of these substances are relatively low, the reactions have not been very sensitive, particularly in those methods in which large volumes of reagents have been used; they have also been unsatisfactory when the sera to be examined have been turbid or jaundiced.

A new reagent, 4:7-diphenyl-1:10-phenanthroline, or bathophenanthroline, was introduced by Case in 1951, and was applied to the estimation of serum iron by Peterson (1953). Its molar extinction coefficient is more than twice that of the previously used reagents. The iron complex formed with it is insoluble in water but soluble in alcohol. Extraction with alcohol can therefore both concentrate the complex and separate it from alcohol-insoluble substances which interfere with the colour, thus increasing the sensitivity of the reagent.

Trinder (1956) has prepared a sulphonated derivative of bathophenanthroline which forms a water-soluble compound with iron and has proved

to be slightly more sensitive than the parent substance.

Peters *et al.* (1956) have described a simple method which can be performed at room temperature, and in which the coloured iron-bathophenanthroline complex is developed in a single phase of water, isopropanol, and isoamyl alcohol. Complete iron recoveries were achieved. When their method was used at room temperature in Cambridge, the final solution was frequently found to be unstable, causing turbidity. This difficulty was overcome by dissolving the bathophenanthroline in ethyl alcohol. It was then decided to find out whether the estimation could be performed more quickly and without loss of simplicity or sensitivity.

Experimental Procedures

Hydrochloric Acid Concentration.—Assuming that the speed of dissociation of the serum iron-protein complex is related to *p*H, and bearing in mind the fact that a proportion of the liberated iron may be lost by adsorption on to insoluble proteins, it was thought reasonable to use the maximum concentration of hydrochloric acid which could be added to serum without precipitating the proteins.

This was determined by adding 1 ml. volumes of hydrochloric acid of increasing normality to 2 ml. volumes of serum at room temperature. The results showed that a final concentration of about 0.6 N hydrochloric acid would be satisfactory.

Protein Precipitation.—Although an excess of trichloracetic acid is necessary to ensure complete precipitation of the serum proteins once the bound iron has been liberated, the total amount of acid which can be used is limited by the necessity of obtaining a minimal final volume, with a *p*H ranging between 4.5 and 4.9. Laurell (1952) pointed out that when hydrazine sulphate, thioglycollic acid, and sodium dithionite are used as reducing substances, this *p*H range is optimal for the rapid conversion of ferric to ferrous ions. It also allows the maximal intensity of colour to be most rapidly developed by the iron-bathophenanthroline complex (personal observation).

The smallest quantity of trichloracetic acid necessary to precipitate the proteins completely was therefore determined by adding varying amounts of 40% trichloracetic acid to mixtures of 2 ml. of serum and 1 ml. of 2 N hydrochloric acid. These were stirred vigorously for ±45 seconds, and centrifuged for 10 minutes at 2,500 r.p.m. Each supernatant solution was decanted, treated with 0.1 ml. of 40% trichloracetic acid, and examined photometrically for the development of a further precipitate. The addition of 0.2 ml. or less of 40% trichloracetic acid was found to produce incomplete precipitation of the proteins.

It was concluded that treatment of 2 ml. of serum with 1 ml. of 2 N hydrochloric acid and 0.5 ml. of 40% trichloracetic acid (producing a final concentration of 0.572 N hydrochloric acid and allowing for an excess of trichloracetic acid) would be likely to extract the bound iron completely.

Recovery Experiments.—The validity of this hypothesis was assessed by means of a series of recovery experiments, using radioactive iron previously bound to the transferrin of the samples employed.

^{59}Fe was obtained from the Atomic Energy Establishment at Harwell, and 0.5 μc., containing not more than 0.4 μg. of elemental iron, was added to 25 ml. of serum and incubated at 37° C. for one hour. The binding of the ^{59}Fe with the transferrin and the absence of free ^{59}Fe was shown in each case by autoradiography after electrophoresis on paper. The radioactivity of the initial samples and of the respective supernatant fractions, obtained after the addition of 2 N hydrochloric acid and precipitation of the proteins with trichloracetic acid, was counted in a well-type scintillation counter. Total counts of 20,000 to 40,000 were obtained from each specimen, and the standard error of counting was found not to exceed 0.7%. The radioactivity of the supernatant

TABLE I
RESULTS OF ^{59}FE RECOVERY EXPERIMENTS

Serum*		HCl		TCA		TGA	Final Concentration of HCl	Final % of TCA	% ^{59}Fe Recovery		No. of Tests
State	Volume	Volume	Normality	Volume	%				Range	Average	
Fresh ..	4	1	2	1·0	40	–	0·33 N	6·66	—	94·0	1
,, ..	2	1	2	1·0	40	–	0·50 N	10·0	99·6–104·2	101·9	3
,, ..	2	1	2	0·5	40	–	0·57 N	5·72	99·0–102·5	100·9	6
,, ..	2	1	2	0·25	40	–	0·615 N	3·07	0	103·0	2
Frozen	2	1	2	1·0	40	–	0·50 N	10·0	94·5–96·7	95·6	2
,,	2	1	2	0·5	40	–	0·57 N	5·72	96·5–96·7	96·6	2
,,	2	1	2	0·5	40	+	0·57 N	5·72	—	101·5	1
,,	2	1	2	1·0	20	–	0·50 N	5·0	—	92·5	1
,,	2	1	6	1·0	20	–	1·50 N	5·0	—	93·6	1
,,	2+4 H_2O	1	2	1·0	40	–	0·25 N	5·0	—	80·9	1
,,†	2+4 H_2O	1	2	1·0	40	–	0·25 N	5·0	90·6–92·0	91·3	2
,,	2	0	0	1·0	40	–	0	13·3	82·9–83·5	83·2	2

* Either serum or plasma. TCA = trichloracetic acid. TGA = thioglycollic acid. † Stood at room temperature for 2 hr. before being centrifuged.

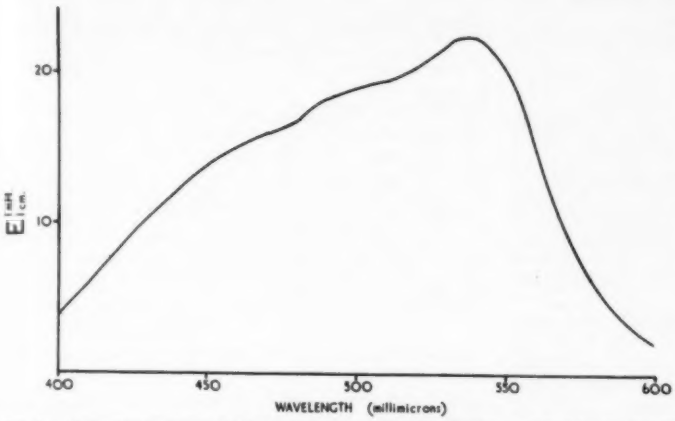

FIG. 1.—Absorption spectrum of the iron-bathophenanthroline complex, obtained by treating the standard iron solution and a reagent blank according to the procedure for serum iron determination described in the text. The optical densities were read in a "unicam" model S.P. 600 spectrophotometer, using 10 mm. cuvettes.

fractions, corrected for dilution, was expressed as a percentage of that of the initial samples, and was considered to represent the recovery of iron. The correction factor described by Severinghaus and Ferrebee (1950) was not used.

These experiments showed that complete recovery of added ^{59}Fe could be obtained from fresh sera* after treatment with 0.5–0.6 N hydrochloric acid and 3–10% trichloracetic acid at room temperature. Previously frozen samples yielded incomplete recovery (92–96%) when treated similarly; recovery was not improved by an increase in the concentration of hydrochloric acid to 1.5 N, but was complete when thioglycollic acid was added to the mixture (Table I).

Adjustment of pH.—The amount of sodium acetate required to buffer the supernatant solution obtained from the mixture of 2 ml. of serum, 1 ml. of 2 N hydrochloric acid, and 0.5 ml. of 40% trichloracetic acid was investigated by adding increasing volumes of a saturated solution of sodium acetate of 2 ml. of the supernatant solution and measuring the *p*H with a glass electrode (Pye model 605 *p*H meter).

A final *p*H of 4.8 was obtained by adding 1 ml. of the sodium acetate to 2 ml. of the supernatant specimens.

Bathophenanthroline Solution. — An excess of bathophenanthroline is necessary in order to combine with all the iron likely to be present, and a concentration of 30% or more of alcohol is required to keep the resulting iron-bathophenanthroline complex in solution at room temperature. The addition of 1.5 ml. of a 0.04% solution of bathophenanthroline in absolute alcohol to the 3 ml. of buffered supernatant solution described above produced a clear solution. The colour was stable for at least 24 hours.

*Several deeply jaundiced samples were used. Completely clear supernatant solutions were obtained.

Photometric Characteristics.—The absorbance of the iron-bathophenanthroline complex was investigated by treating 2 ml. volumes of increasing concentrations of a solution of ferric chloride in a similar manner. The curve was found to be linear to a concentration of just over 0.76 μg. Fe per ml. of the final solution, corresponding to 300 μg. Fe per 100 ml. of the ferric chloride solution. The absorption spectrum showed a peak at 534 to 538 mμ (Fig. 1), and the millimolar absorbance at 536 mμ was found to be 22.4.

Proposed Method

Iron-free Water.—Water for the preparation of all solutions and the rinsing of all acid-washed apparatus was distilled twice over a glass and passed through a de-ionizing resin column† before use.

Apparatus.—All the glass used, including the reagent bottles, was washed in a detergent solution, soaked in 50% hydrochloric acid for a minimum of 24 hours before use, and finally rinsed in iron-free water. Stainless steel needles were washed through with iron-free water.

A rubber bulb-type pipette filler ("hoslab" propipette‡) was used for accurate pipetting when necessary.

Reagents.—The following were used:

2 N Hydrochloric Acid.—Concentrated hydrochloric acid, 200 ml. (AnalaR, sp.g. 1.18) was diluted to 1 litre with water.

40% Trichloracetic Acid.—This was distilled to remove traces of iron.

Thioglycollic Acid.—This was redistilled to remove traces of iron, which vary in amount in different specimens.

Saturated Solution of Sodium Acetate.—This was prepared from sodium acetate trihydrate (AnalaR). Iron contamination was removed by precipitation of the sodium acetate in ethyl alcohol in the cold, after 10 ml. of the bathophenanthroline reagent had been added to a super-saturated solution of 500 g. at 37° C.

4 : 7-Diphenyl-1 : 10-phenanthroline (Bathophenanthroline).—A 0.04% solution was made in ethyl alcohol (AnalaR).

Standard Iron Solutions.—One hundred milligrams of freshly cleaned pure iron wire were dissolved in

†Elgastat, Type B102, Elga Products Ltd., London, S.W.19.
‡Hospital and Laboratory Supplies Ltd., 12, Charterhouse Square, London, E.C.1.

4 ml. of 50% hydrochloric acid, and diluted to 1 litre with water, to form a stock solution.

A dilute working solution, containing 1 μg. Fe/ml., was prepared.

Procedure.—This is as follows :

(a) For Values Not Exceeding 300 μg. per 100 ml.—Two drops of thioglycollic acid were added to 2 ml. of serum in a 10 ml. conical centrifuge tube. The mixture was shaken briefly and 1 ml. of 2 N hydrochloric acid was then added, with further shaking. Next 0.5 ml. of 40% trichloracetic acid was added; the contents of the tube were stirred vigorously with a glass rod for ±45 seconds, and then centrifuged for 10 minutes at 2,500 r.p.m.

Two millilitres of the supernatant fluid were pipetted into a suitably stoppered tube, 1 ml. of saturated sodium acetate and 1.5 ml. of the bathophenanthroline reagent added, and the contents mixed by swirling. Iron standards containing 1 and 2 μg. Fe per 2 ml. respectively and a reagent blank using 2 ml. of water instead of serum were prepared in a similar manner.

(b) For Values Above 300 μg. per 100 ml.—One millilitre of serum, 0.5 ml. of 2 N hydrochloric acid, 0.25 ml. of 40% trichloracetic acid, 1 ml. of the supernatant solution, 0.5 ml. of saturated sodium acetate, and 3 ml. of the bathophenanthroline reagent were substituted for the quantities used above.

After full colour development had taken place (15–30 sec.) the optical densities of the serum extracts and standard iron solutions were read against the reagent blank in a "unicam model" S.P. 600 spectrophotometer at 536 mμ, using 10 mm. fused glass cuvettes. The iron values were then read off from a previously calibrated graph prepared by plotting optical densities against concentrations of the standard iron solution. Any suitable photometric apparatus can be used instead of the above instrument.

The following optical density readings were obtained with a solution containing the equivalent of 100 μg. of iron per 100 ml., using cells of various types.

Cell	Final Volume (ml.)	Optical Density
1·1 cm. tube	4·5	0·095
10 mm. cuvette	4·5	0·1025
20 ,, ,,*	4·5	0·2025

*Raised 0.5 cm. in the cuvette holder.

It is preferable to use 20 mm. cuvettes, so that the optical density readings may be brought within the range of the optimal sensitivity of the instrument.

Comparative Readings

The efficiency of the method was contrasted with that of Peters *et al.* (1956), by carrying out a series of four replicate analyses on three previously frozen samples of serum, which contained low, average, and high amounts of bound iron respectively. The values obtained are listed in Table II and show good agreement and reproducibility.

TABLE II

COMPARISON OF IRON VALUES OBTAINED BY PRESENT METHOD AND THAT OF PETERS *ET AL.* FROM THREE SAMPLES OF SERUM

Serum Sample	Iron Values (μg.) per 100 ml.	
	Present Method	Peters *et al.*
I	72 72 72 71	70 70 69 70
II	121 121 121 121	120 120 119 120
III	192 192 190 190	191 186 188 186

Discussion

Peters *et al.* (1956) have extracted iron completely from fresh and from previously frozen sera, whether turbid or jaundiced, by using 0.12 N hydrochloric acid for 30 minutes at room temperature in the presence of thioglycollic acid.

Using 0.67 N hydrochloric acid at room temperature, with minimal incubation, Bothwell and Mallett (1955) also obtained complete recovery of iron. This was done in the absence of thioglycollic acid, which, in their method, was only added to the supernatant fluid obtained after precipitation of the proteins with trichloracetic acid. Ramsay (1957) employed their procedure to investigate the effects of varying concentrations of hydrochloric acid acting on serum for different lengths of time, and was unable to confirm their results. He found that 0.67 N hydrochloric acid resulted in incomplete iron extraction after 60 minutes' incubation at room temperature and that 1.0 N hydrochloric acid needed 20 minutes' incubation, at room temperature, to produce full recovery of iron.

The present studies have demonstrated that 0.6 N hydrochloric acid, as used in Bothwell and Mallett's procedure, does extract iron completely from fresh sera at room temperature and in the absence of a reducing agent, but that incomplete recoveries are obtained from stored specimens, unless the iron-protein complex is dissociated in the presence of thioglycollic acid.

Although not recorded, it is possible that Bothwell and Mallett may have used fresh sera

and that Ramsay may have used stored samples in their respective investigations. If so, this might well account for the apparent contradictions stated above.

The sensitivity of the proposed method is slightly less ($\pm 5\%$) than that of Trinder's (1956) procedure, but is achieved without the complication of sulphonating the bathophenanthroline and of heating the reagent mixture. The sulphonated derivative has not yet been isolated as a definite substance of known structure and so it is difficult to account for its increased sensitivity other than by postulating an increase in the number of resonance structures.

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THE DETERMINATION OF SERUM IRON AFTER THE INTRAVENOUS INJECTION OF IRON-DEXTRAN

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A rapid technique for the determination of iron in serum, after the intravenous injection of iron-dextran, is presented.

Dithionite is used for the reduction of ferric to ferrous iron.

The use of a detergent solution enables protein precipitation to be omitted.

The iron reagent, 4:7-diphenyl-1:10-phenanthroline, and its ferrous complex are both soluble in the dilute detergent solution.

Lipaemic serum can be used without affecting the results.

The recovery of added ferric iron, as iron-dextran, is quantitative.

The introduction by MacKenzie and Tindle (1959) of an iron-dextran compound for the measurement of plasma volume has increased the necessity for an accurate and rapid technique for serum iron determination in the concentration range 500–2,500 μg. Fe/100 ml. The determination of serum iron described by MacKenzie and Tindle is a modification of the method of Trinder (1956) which involves protein precipitation before colour development with the sulphonated 4:7-diphenyl-1:10-phenanthroline (bathophenanthroline) reagent. In an attempt to simplify this technique it was decided to investigate the possibility of omitting protein precipitation. If this could be achieved time would be saved, smaller volumes of serum would be required, and the risk of contamination would be reduced since the determination would be completed in one test-tube.

Dilute solutions of detergents were found to be effective in preventing precipitation of the serum proteins during colour development at a temperature of 90 to 95° C. Despite high protein concentrations and prolonged boiling, perfectly clear solutions were obtained. The commonly used detergent "teepol" (Shell Chemical Limited) was found to be very satisfactory and has been used in this investigation. The bathophenanthroline reagent and its red ferrous complex, both normally insoluble in aqueous solution, are readily soluble in dilute solutions of the detergent; sulphonation with chlorosulphonic acid was therefore unnecessary. The method is rapid and accurate and can be done routinely in any clinical laboratory.

Method

Apparatus and Reagents.—All apparatus should be rendered iron-free by any of the conventional methods. Thereafter the test-tubes simply require rinsing in distilled water after each batch of determinations. Although the "teepol" used was ordinary commercial grade as supplied for cleansing purposes it was surprisingly free from iron and required no preliminary treatment. Teepol powder ("teepol XL") can also be obtained, but this contains considerably more iron than the liquid concentrate and was not used in this study. The distilled water used throughout was rendered iron-free by passage through an "elgalite" ion-exchange resin. The reagent blank was of the order of 0.2 μg. Fe/determination, but about half of this "iron" was due to the colour of the iron reagent.

Iron Reagent.—Dissolve 5 mg. 4:7-diphenyl-1:10-phenanthroline in 10 ml. liquid "teepol" concentrate (27% active ingredient) by warming on a hotplate and breaking down any lumps with a glass rod. After cooling make the volume to 100 ml. with distilled water. When larger volumes of iron reagent are required the preparation of a stock solution containing 50 mg. bathophenanthroline/100 ml. "teepol" concentrate is recommended. The working reagent is obtained by diluting this stock solution 1 in 10 with water. The reagent appears to keep indefinitely.

Reducing Agent.—Solid sodium hydrosulphite (dithionite) $Na_2S_2O_4 + H_2O$, which should be of the highest available purity.

Standard Iron Solution (1 mg. Fe/ml.).—Dissolve 702 mg. A.R. grade ferrous ammonium sulphate ($(NH_4)_2SO_4.FeSO_4.6H_2O$) in N/10 sulphuric acid and make the volume to exactly 100 ml. with the acid.

Procedure.—Treat both blank and test sera (or heparinized plasma) as follows:

Deliver 0.2 ml. serum into a 15 ml. "pyrex" test-tube containing 3.8 ml. iron reagent and 10–20 mg. dithionite.

Mix by lateral shaking and place in a water-bath at 90–95° C. for three minutes.

Remove and cool to room temperature.

Read the optical density of the test solution against the blank in the spectrophotometer at 530 mμ.

Standards.—Prepare working standards by diluting the standard iron solution (1 mg. Fe/ml.) 3 in 200 and 3 in 100 with distilled water to give solutions containing 15 and 30 μg. Fe/ml. Treat 0.2 ml. of these diluted standards (S_1 and S_2) exactly as described for serum or plasma and read the optical densities of the resulting solutions in the spectrophotometer against a reagent blank. As there is complete agreement with Beer's law the optical density of S_2 should be exactly twice that of S_1. Using a "unicam" SP. 600 spectrophotometer at 530 mμ and with 10 mm. cells, an optical density of 0.092/μg. Fe was obtained.

Calculation.—This is as follows:

$$\text{Iron content of test} = \frac{\text{O.D. of test}}{\text{O.D. of } S_1} \times 15, \text{ or } \frac{\text{O.D. of test}}{\text{O.D. of } S_2} \times 30 \text{ μg./ml.}$$

For instruments which require up to 5 ml. of final solution the volume of iron reagent may be increased to 4.8 ml.

Iron Injected.—To determine the exact amount of iron injected, dilute the injection solution with distilled water to contain approximately 20 μg. Fe/ml. Treat 0.2 ml. of this solution exactly as described for serum or plasma and read in the spectrophotometer against a reagent blank to obtain μg. Fe/0.2 ml. solution.

Then

$$\text{Iron injected per ml.} = \frac{\text{μg. Fe/0·2 ml.} \times 5 \times \text{dilution factor}}{1{,}000} \text{ mg.}$$

The plasma volume can now be calculated in the usual way by dividing the μg. iron injected by the difference in iron content of the blank and test sera.

$$\text{Plasma volume} = \frac{\text{μg. iron injected}}{\text{iron content of test sample (μg./ml.)}} \text{ ml.}$$

Results

Recovery experiments were carried out by adding known amounts of iron, in the form of iron-dextran, to pooled serum. Analysis of the resulting sera by the proposed method showed that the recovery was quantitative (Table I).

TABLE I

RECOVERY OF IRON ADDED TO SERUM

Iron Added (μg. Fe/100 ml. Serum)	Iron Found (μg. Fe/100 ml. Serum)	% Recovery
500	515	103
1,000	1,000	100
1,500	1,480	98·7
2,000	2,050	102·5
2,500	2,490	99·6

TABLE II

RESULTS OF DUPLICATE DETERMINATIONS OF IRON IN SERUM SAMPLE

Optical Density		Serum Sample (μg. Fe/ml.)	Optical Density		Serum Sample (μg. Fe/ml.)
Standard (20 μg. Fe/ml.)	Serum Sample		Standard (20 μg. Fe/ml.)	Serum Sample	
0·377	0·338	17·93	0·377	0·336	17·83
	0·336	17·83		0·331	17·56
0·380	0·334	17·58	0·373	0·338	18·12
	0·336	17·68		0·338	18·12
0·379	0·339	17·89	0·378	0·339	17·94
	0·341	18·00		0·338	17·88

Haemolysed serum cannot be used since the use of dithionite would include haem iron in the result. The use of grossly lipaemic serum does not interfere with the clarity of the final solution. To demonstrate this a 1 in 20 dilution of a lipaemic serum in physiological saline was prepared. This gave an optical density of 0.490 at 530 mμ; a 1 in 20 dilution of the same serum in 10% v/v teepol gave an optical density of 0.025 at the same wavelength. The serum used in this experiment was considerably more lipaemic than would normally be encountered in plasma volume measurements.

To demonstrate the reproducibility of the technique a number of duplicate determinations of the iron content of a serum sample were made on different occasions using a freshly prepared iron standard (20 μg./ml.). The results are shown in Table II.

Discussion

Under the conditions of the test the bathophenanthroline reagent is specific for ferrous iron. Buffering of the reagent was considered unnecessary since the final solution is maintained at an acid *p*H by the addition of the dithionite; colour development is maximum over the *p*H range 3.5–7.0.

If the iron content of the test serum is low the volume of serum used can be increased to 0.5 ml., 3.5 ml. of the iron reagent then being used. With an injection of 50 mg. iron and a plasma volume of 3 litres the optical density of the final test solution will be about 0.30 using 0.2 ml. serum.

The reduction of ferric to ferrous iron with dithionite is complete within two minutes at room temperature, but heating at 90 to 95° C. is necessary to render the final solution optically clear. Due to a slight turbidity unheated serum solutions have a higher optical density than the corresponding heated solution, and this difference decreases slowly during the first hour after mixing. The use of dithionite reduces the time for a plasma volume estimation to little more than 30 minutes from the time of injection, if heparinized

plasma is used. Milder reducing agents, such as sodium sulphite which do not include haem iron in the result, require heating at 90 to 95° C. for about an hour to obtain a quantitative recovery of iron. Such a procedure does not recommend itself for inclusion in a rapid technique. The use of solid dithionite is perfectly satisfactory since a good grade product contains as little as 0.3 μg. Fe/g.; the addition of 100 mg. solid, five times the recommended amount, would increase the iron content of the final test solution by less than 1%. The addition of 0.05 ml. of a saturated solution (1 g. in 4 ml. water) of dithionite per determination is equally satisfactory, but the solution must be prepared immediately before use.

The proposed method compares favourably in accuracy with the method used by MacKenzie and Tindle, but the time for a single determination has been reduced by half. Recovery experiments were considered satisfactory.

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I wish to thank Dr. P. Trinder for much useful advice.

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AN IMPROVED WHITE CELL DILUENT FOR USE WITH THE EEL ELECTRONIC BLOOD CELL COUNTER

BY

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An improved white cell diluent for use with the Eel electronic counter is described. It possesses advantages over previously described diluents in the rapidity of its action as a red cell stromalysin and in its ability to conserve surviving leucocytes for long periods of time. These properties enable counts to be made either immediately after preparation of the suspension or several hours later. The diluent is equally suitable for use with capillary or venous blood samples. When used for counting leucocytes it has been found necessary to effect a minor modification to the machine whereby the light intensity is reduced by approximately one-half.

The advantages of using electronic aids in the counting of red cells have been clearly established for some time, but as yet the counting of white cells has proved much less satisfactory because of difficulty in finding a suitable diluent. After several months' trial with previously recommended diluents for the Eel and other electronic counters it became clear that none of them was ideal for routine use with the Eel counter, and accordingly an investigation was undertaken with the object of developing a white cell diluent which would conform to the following requirements: (*a*) It should be equally adaptable for both visual and electronic counting of leucocytes. (*b*) It should be capable of lysing red cells quickly so that counts could be made immediately after the suspension has been prepared. (*c*) The cell suspension should remain stable for at least 12 hours so that counts could be made several hours after preparation. (*d*) It should be equally adaptable for both capillary and venous blood samples.

After experimenting with a large number of different lysing agents we believe we have found a diluent which fulfils these criteria. It has also been our experience that the efficiency of the Eel machine in counting leucocytes is much improved by making a minor modification to the dark-field illumination.

Preparation of Suspension

The following stock solution of diluent is prepared freshly each week:

Cetrimide 40% ("cetavlon" I.C.I.) ..	12.5 ml.
Glacial acetic acid	5.0 ml.
Formalin	10 ml.
Aqua dist. ad	1,000 ml.

From this stock solution a volume sufficient for one day's use is filtered through sintered glass into a scrupulously clean glass container stoppered with rubber or glass. The importance of avoiding metal stoppers is emphasized, as it has been found that the diluent exerts a chemical action upon aluminium, causing the release of particles which may seriously affect the accuracy of the count. It is also important to avoid contamination by dust or other particles which may either interfere with the efficiency of the jets or register as leucocytes.

Cetrimide in acid solution has been found to be a very effective stromalysin, and leucocytes may be counted within seconds of mixing with the diluent, and remain unaffected for at least 24 hours at room temperature. If the mixture is stored at 4° C. accurate visual and electronic counts may be made after the lapse of several days. The solution is equally suitable for use with venous blood treated with "sequestrene" (E.D.T.A.) or capillary blood pipetted directly into the diluent. Oxalated blood is not suitable for use in the electronic counting of leucocytes. In common with other detergents cetrimide tends to froth when shaken vigorously; in practice this property has been found not to impair the accuracy of the counts in any way.

Various strengths of cell suspension have been used in this investigation, and it has been found that a dilution of 1 in 20 is the most satisfactory for routine use. Venous or capillary blood, 0.05 ml., is pipetted into 0.95 ml. diluent and transferred to a small-bore sampling tube of approximately $\frac{1}{4}$ in. diameter. In order to avoid the introduction of cell debris from the skin of the finger the tubes are sealed with a clean rubber stopper before mixing. Counts may be made after the lapse of a few seconds or left overnight at room temperature and counted on the following morning.

Modification to Counter

For the accurate counting of white cells the sensitivity of the machine must be reduced to a point where small aggregates, such as red cell debris, are not registered on the dark-ground field. Small particles of this size are normally included in the counting of red cells, but their number in relation to the total count is so small that their inclusion does not lead to any significant error. In the counting of a much smaller number of leucocytes this error cannot be permitted, and it is accordingly necessary to reduce the sensitivity of the machine by the simple device of reducing the intensity of the light source. This may be most easily accomplished by inserting a suitable resistance (approximately ¼ ohm) in the light circuit, or more simply by impeding the light path by the introduction of a neutral grey screen or iris diaphragm. It has been our experience that a reduction of approximately 50% in the light intensity permits the accurate counting of white cells over a

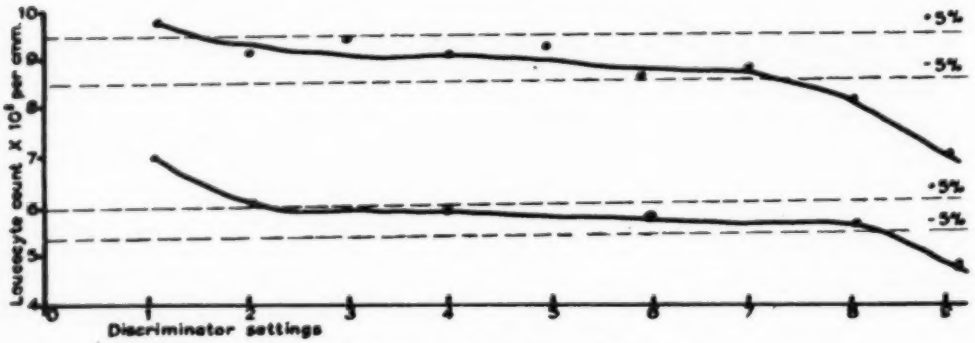

FIG. 1.—Typical plateaux of leucocyte counts (dilution 1 in 20).

TABLE

RELATIONSHIP BETWEEN 30 VISUAL AND ELECTRONIC LEUCOCYTE COUNTS AT VARYING DILUTIONS

Dilution	Visual Count		Machine Count					
	Cells Counted	Total Count × 10^3	Mean of 5 Counts × 10^3	Cells Counted				
				1st	2nd	3rd	4.h	5th
1 in 10	395	9·87	9·92	2,941	3,012	3,057	2,956	2,927
1 ,, 10	398	9·95	9·31	2,848	2,751	2,848	2,753	2,770
1 ,, 10	244	6·10	5·97	1,779	1,742	1,829	1,797	1,820
1 ,, 10	487	12·17	12·69	3,854	3,786	3,838	3,756	3,809
1 ,, 10	329	8·22	7·99	2,457	2,359	2,400	2,424	2,353
1 ,, 10	315	7·87	7·61	2,313	2,347	2,240	2,252	2,274
1 ,, 10	244	6·10	6·26	1,808	1,899	1,962	1,912	1,809
1 ,, 10	441	11·02	11·51	3,559	3,467	3,347	3,448	3,445
1 ,, 10	445	11·12	11·62	3,625	3,419	3,611	3,324	3,458
1 ,, 10	514	12·85	13·19	3,905	4,061	3,886	3,952	3,982
1 ,, 20	308	6·84	7·07	1,067	1,025	1,058	1,091	1,067
1 ,, 20	340	7·55	7·61	1,132	1,151	1,089	1,167	1,171
1 ,, 20	495	11·00	10·95	1,646	1,708	1,641	1,607	1,615
1 ,, 20	274	6·09	5·58	851	874	847	779	837
1 ,, 20	469	10·42	10·82	1,604	1,572	1,646	1,657	1,641
1 ,, 20	275	6·11	6·59	1,017	986	993	968	979
1 ,, 20	796	17·68	17·32	2,455	2,692	2,600	2,600	2,643
1 ,, 20	464	10·31	10·78	1,606	1,646	1,610	1,633	1,603
1 ,, 20	350	7·77	7·81	1,146	1,195	1,187	1,187	1,143
1 ,, 20	419	9·31	9·94	1,484	1,521	1,519	1,444	1,460
1 ,, 50	727	11·36	11·61	661	690	702	728	703
1 ,, 50	352	5·50	5·84	368	366	330	347	342
1 ,, 50	385	6·01	6·74	400	442	360	427	394
1 ,, 50	841	13·14	12·03	751	751	640	713	755
1 ,, 50	640	10·00	10·09	606	601	617	569	634
1 ,, 50	943	14·73	15·27	895	872	924	906	985
1 ,, 50	386	6·03	5·81	371	315	343	388	326
1 ,, 50	475	7·42	7·56	429	439	451	454	497
1 ,, 50	492	7·68	7·83	457	431	472	498	493
1 ,, 50	706	11·03	10·89	631	665	699	607	665

range of discriminator settings comparable to that normally achieved in counting red cells on the unmodified machine (Fig. 1).

Comparison with Visual Counts

The accuracy of the mechanical counter has been verified by comparison with 264 visual counts which formed a sequential series of routine white cell counts performed in the haematology department. In all cases the visual counts were done before the electronic, and both counts were carried out on the same cell suspension. Each machine count was computed as the mean of three readings. In 209 cases a cell suspension diluted 1 in 50 was used, and in 55 cases a 1 in 20 dilution was employed. This investigation showed that 62.9% of the electronic counts fell within ± 1 standard deviation of the visual counts, 92% fell within ± 2 s.d., and 99.2% fell within ± 3 s.d.

A second investigation was then carried out with the purpose of reducing the inherent error in both the visual and electronic methods by counting a larger number of cells than is usual in the routine technique. Thirty samples of blood were visually counted for their leucocyte content, the cases being divided into three groups of 10 samples, diluted 1 in 10, 1 in 20, and 1 in 50. The total number of cells visually counted in each case varied from 274 to 943. From the same suspensions electronic counts were then made. Each count was repeated five times, and the mean of each count was computed from three samplings so that each suspension was fed into the machine 15 times. The results of this investigation are shown in the Table, where it will be seen that very close correlation between the visual and electronic counts was achieved.

Discussion

Several diluents for the electronic counting of white cells have been described, but all seem to possess disadvantages of one sort or another. Boxall (1959) suggested the use of the non-ionic detergent "nonidet" P.40, but not only does this solution become turbid, due to precipitated surfactant, on slight rises of temperature, but the suspension is unstable, necessitating counting the white cells within half an hour of preparing the suspension. Richar and Breakell (1959), working with the Coulter counter, recommended saponin as a lysing agent. This diluent also possesses serious disadvantages, for not only must counts be completed within one hour of preparation but occasional agitation must be carried out for 10 to 15 minutes before counting. We have ourselves used various saponin-containing diluents and have found them all greatly inferior to cetrimide acetic acid diluent. Akeroyd *et al.* (1959) have recently recommended "triton X 100" as a lysing agent, but here also the cytolytic effect on leucocytes was found to be too rapid to allow of a reasonable interval between preparation of the cell suspension and counting the cells (Richar and Breakell, 1959).

The cetrimide acetic diluent recommended here does not appear to possess any of these disadvantages, for while stromalysis of red cells is almost immediate its cytolytic effect on white cells is not apparent for at least 24 hours when kept at room temperature.

A recently developed wetting agent, "hyamine 2389," prepared by the Rohm and Haas Co. for use with chemical auto analysers, has been tested for its suitability as a white cell diluent, and preliminary investigations suggest that it possesses many of the advantages of cetrimide.

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SERUM TRANSAMINASES AND ISOCITRIC DEHYDROGENASE IN KWASHIORKOR

BY

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Serum glutamic oxalacetic transaminase (G-OT), glutamic-pyruvic transaminase (G-PT), and isocitric dehydrogenase (ICD) have been estimated in six groups of subjects, namely, normal British adults, normal Nigerian adults, undernourished Nigerian adults and children, Nigerian children with florid protein malnutrition (kwashiorkor), and cases of protein malnutrition after treatment.

The normal range in Nigerian adults was apparently slightly lower than in European adults.

A marked increase in serum enzyme activity was found in florid malnutrition, and a slight rise in undernutrition. In all cases serum G-PT showed lesser changes than G-OT and ICD.

The causes of these abnormalities are discussed. The increased serum enzyme activity is thought to be derived primarily from the liver and possibly also from voluntary muscle. Estimation of serum enzymes is recommended in the investigation of protein malnutrition.

In West Africa protein malnutrition presents in children as kwashiorkor. Amongst the established pathological findings in kwashiorkor are fatty liver and muscle wasting.

Determination of the activities in serum of enzymes which take part in cellular metabolic processes provides tests of cellular damage, particularly in the liver, which are more sensitive than any other laboratory procedures. This damage may be altered selective permeability, and does not necessarily imply necrosis. It was thought that these serum estimations, in particular of glutamic-oxalacetic transaminase (G-OT), glutamic-pyruvic transaminase (G-PT), and isocitric dehydrogenase (ICD), might detect hepatocellular damage in cases of protein malnutrition before other biochemical or clinical signs of liver involvement could be found. During a visit to the Chemical Pathology Department of University College Hospital, Ibadan, Nigeria, in the autumn of 1958 this problem was investigated, and an attempt was made to correlate the results with the general biochemical survey of kwashiorkor proceeding in that department.

Clinical Material

(A) **European Controls (22 Subjects).**—They were normal subjects, in the same age group as the Nigerian normals, who had previously been studied at the Royal Free Hospital.

(B) **Normal Adults (14 Subjects).**—They were healthy Nigerian medical students, aged 20–30 years. They came from all parts of the country and were eating a mixed diet which contained about 70 g. protein daily. No normal children were examined in this series.

(C/D) **Undernourished Adults (Seven Subjects) and Children (10 Subjects).**—They were living in an agricultural village (Ilora) about 10 miles from Ibadan, which was undergoing a nutritional survey, and I am grateful to Dr. Alexander Boyo for his collaboration. The adults were aged 25–40 and the children 5–7. They had no evidence of illness and were living a normal life; they were not anaemic and had normal serum protein levels. The daily protein intake of the adults was about 40 g.: that of the children is not known, but was probably less.

(E) **Children with Protein Undernutrition (Kwashiorkor) (11 Subjects).**—They were in-patients or out-patients of University College Hospital, Ibadan, with a history of a very low protein diet, who had clinical symptoms of kwashiorkor of various grades of severity, and a total serum protein of less than 5 g./100 ml. The age distribution was 1–6 years, and they were in general a few years younger than the undernourished children.

(F) **Children with Recovered Protein Undernutrition (Nine Subjects).**—They were out-patients of University College Hospital, Ibadan, who had recently finished their course of treatment for kwashiorkor (a high-protein diet), were clinically recovered, and their total serum protein was more than 6.5 g./100 ml.

Methods

Serum G-OT was measured by the method of Karmen (1955), serum G-PT by the method of Wróblewski and LaDue (1956), serum ICD by the method of Wolfson and Williams-Ashman (1957), all with minor modifications, e.g., most analyses were performed with 0.2 ml. serum. The question of units is important (Daly and Jordan, 1959). It is possible to express results of serum enzyme analyses either in spectrophotometric units (for G-OT, Karmen, 1955; for G-PT, Wróblewski and LaDue, 1956; for ICD, Kerppola, Nikkilä, and Pitkänen, 1959), or in substrate units (for G-OT, Hauss and Leppelmann, 1958; for G-PT, Dreyfus, Schapira, and Schapira, 1958; for ICD, Wolfson and Williams-Ashman, 1957). Spectrophotometric units are easier to calculate, as a simple factor of the change in optical density which occurs during the estimation, provided that conditions are standard. They have been almost universally used for G-OT and G-PT. Substrate units, which represent quantities of reactant used or formed, are enzymologically more correct and are perhaps more commonly used for ICD. The optimum temperature to which results should be corrected is also undecided (Baron and Bell, 1959a). In this work spectrophotometric units, one unit being 0.001 change in $O.D._{340}$/ml. serum in 3 ml. reaction volume/minute at 25° C. (as this was the temperature of the air-conditioned laboratory), were employed for all three enzymes; for ICD, 1 spectrophotometric unit = 30 reactant units.*

In the routine chemical pathology laboratory, serum proteins were estimated by the method of Wolfson, Cohn, Calvary, and Ichiba (1948), serum bilirubin by the method of Powell (1944), and serum cholesterol by the method of Sperry and Webb (1950). The normals for Nigerian children are: total protein 6–8.5 g./100 ml., albumin 2.5–4.0 g./100 ml., bilirubin 0.3–1.0 mg./100 ml., total cholesterol 100–180 mg./100 ml., percentage free cholesterol 20–40% (Edozien, personal communication).

*In a previous communication (Baron and Bell, 1959b), ICD units were expressed per ml. serum/1.5 ml. reaction volume: this unit introduces confusion and has been abandoned. A recent international subcommission has recommended that all enzyme units be expressed as millimole substrate utilized/minute/unit volume (? ml. or l., King, E. J., personal communication).

Results

In the transaminase assays I observed rapid utilization of D.P.N.H. before the addition of oxoglutarate in the analysis of certain sera from children of groups C, D, and E. This was not found in the sera of adults, and was not apparently related to any other biochemical finding. It was considered that a high concentration of plasma

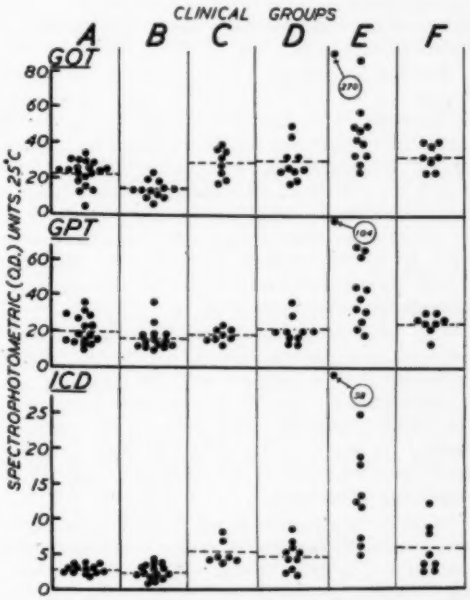

FIG. 1.—Chart showing results of serum G-OT, G-PT, and ICD analyses in six clinical groups. (A) Normal European adults, (B) normal Nigerian adults, (C) undernourished Nigerian adults, (D) undernourished Nigerian children, (E) active kwashiorkor, (F) recovered kwashiorkor. (The means are expressed in all groups except E by horizontal dashes.)

TABLE I
SERUM ENZYME ACTIVITIES AND RESULTS OF OTHER SERUM ANALYSES IN CHILDREN WITH PROTEIN MALNUTRITION (KWASHIORKOR)

Case No.	Albumin (g./100 ml.)	Total Protein (g./100 ml.)	G-OT (units/ml.)	G-PT (units/ml.)	ICD (units/ml.)	Total Bilirubin (mg./100 ml.)	Total Cholesterol (mg./100 ml.)	% Free Cholesterol
M 22	0·4	3·4	58	66	12	0·6	265	40
M 7	0·4	4·2	43	20	5	0·6	—	—
M 17	0·9	3·9	27	45	5	3·0	—	—
M 15	0·9	4·0	49	21	6	0·6	—	—
M 8	0·9	4·4	35	44	25	—	171	67
M 13	0·9	4·8	94	33	18	0·3	—	—
M 11	1·3	3·9	270	104	38	1·8	305	90
M 6	1·4	4·6	24	62	7	—	—	—
M 3	1·4	4·8	35	65	19	0·6	240	60
M 25	1·8	3·8	49	31	—	1·2	—	—
M 5	1·8	5·0	46	26	12	—	230	50
M 16	2·0	3·9	40	39	14	0·6	—	—

pyruvate could at least in part account for this finding, but because of lack of time I was not able to investigate this further. This has been confirmed by later work (Edozien, 1959).

Fig. 1 is a comparison of the results of the serum enzyme estimations on all groups of subjects. The diagram shows, as well as the scatter, the mean for all groups except E where the considerable scatter would make it irrelevant. The small numbers involved, and the asymmetric grouping, make statistical analysis unwise. However, inspection of the results yields useful information.

Normal Europeans (A) seem to have slightly higher values for the three serum enzymes in comparison with normal Nigerians (B). It is doubtful whether any significance can be attached to this, but it would be interesting if this observation were confirmed on larger numbers. If so, it would not be the only biochemical parameter to be different in healthy west Africans and healthy west Europeans (Edozien, 1958): for example, which may be relevant, serum albumin is lower in Nigerians than in Europeans.

The undernourished adults (C) have serum enzyme activities that are generally moderately increased, but the increase in G-PT is small. The increase in G-OT and ICD is more apparent, and the mean values are about twice those found in normal subjects. The values found in undernourished children (D) are similar to those of the undernourished adults.

In children with established protein malnutrition (E) a marked increase in serum enzyme activity (over D) is usually found. Again G-PT shows the smallest changes, and some results are not increased. The proportionate increase of ICD activity is probably greater than of G-OT activity. Table I gives the results of the serum enzyme estimations compared with results of other biochemical analyses in this group. The cases are arranged in order of the serum albumin concentration, which serves as an arbitrary index of the degree of malnutrition. There is no apparent correlation between the enzyme activities and any other biochemical abnormalities. The subject (M 11) with the most abnormal enzymes results did have the most abnormal cholesterol and was jaundiced but did not have the grossest hyperproteinuria, whereas M 17, who was severely jaundiced, had very little change in serum enzymes.

In the cured children (F) the serum enzyme activities show similar values to those found in the undernourished children (D) of their own home environment.

Discussion

In protein malnutrition two opposing factors could affect serum enzyme activities. As enzymes are proteins, the general protein deficiency could also affect the synthesis of the serum enzymes; low values for serum alkaline phosphatase, correlating with low serum albumin, have been reported in kwashiorkor (Schwartz, 1956). On the other hand, tissue wasting and cell damage could increase the serum concentrations of the intracellular enzymes. The increase in serum enzymes found in kwashiorkor could come from erythrocytes, cardiac muscle, voluntary muscle, or liver.

Haemolysis causes an increase in serum ICD, but very little in G-OT or G-PT. Care was taken in collecting and separating the blood, and any haemolysed samples were rejected.

It is established that in myocardial necrosis, when the serum G-OT is high, the G-PT is normal or slightly raised (Wróblewski, 1958) and the ICD is also normal (Baron and Bell, 1959b). The abnormal enzyme pattern of myocardial damage is quite different from that found here in protein malnutrition.

Wasting of voluntary muscle is marked in kwashiorkor. Although muscle wasting in starvation is not associated with a rise in serum aldolase or lactic dehydrogenase (White, 1958), yet these enzymes, G-OT, and to a lesser extent G-PT, show increased serum activity in muscle disease (Dreyfus, Schapira, and Schapira, 1958). The concentration of ICD in voluntary muscle is low, and in muscular dystrophy, where other serum enzymes are markedly raised, serum ICD is normal (White, 1958). In a small personal series of cases of muscle dystrophy and myopathy, with raised serum G-OT, the serum ICD was normal (Baron and Bell, unpublished observations). This evidence suggests that skeletal muscle damage is not the principal cause of the rise in serum enzymes in kwashiorkor.

The abnormal enzyme pattern in this series is the same as that found in hepatocellular damage in which serum ICD, G-OT, and G-PT are all increased, though there may be variations in the proportionate increases in the different enzymes in different types of liver disease (Sterkel, Spencer, Wolfson, and Williams-Ashman, 1958; Wróblewski, 1958; Kerppola *et al.*, 1959). The evidence supports the view that the alterations in serum enzymes in protein malnutrition are due primarily to release of enzymes from liver cells, though general tissue breakdown, principally in the skeletal muscles, may play a part.

As different "liver function tests" measure different functions of the liver, the lack of correlation between the abnormal enzyme results and the results of the other liver function tests is not surprising, and occurs in other liver diseases. In patients with kwashiorkor dying at the height of the illness the main structural change in the liver is deposition of fat, which is more marked than cell necrosis. In chlorpromazine jaundice there is an increase in serum G-OT without apparent liver cell necrosis, and it has been suggested that this is caused by altered cell permeability (Shay and Siplet, 1957): the same may apply in kwashiorkor. In their studies in Guatemala on the biochemical changes in the liver of kwashiorkor, Burch, Arroyave, Schwartz, Padilla, Béhar, Viteri, and Scrimshaw (1957) showed that an average of 30% of liver protein was lost, and that (of the enzymes studied here) the concentration of G-OT relative to protein was unchanged: their results indicate that liver G-OT is lost in parallel with liver protein.

As a result of the studies it is suggested that estimation of serum enzymes may be a useful additional tool in the assessment of cases of incipient or florid protein malnutrition. Serum G-OT is at present the recommended enzyme to assay, as the changes are almost as consistent as in serum ICD, but a simple and relatively cheap colorimetric method of assay is available. Colorimetric assay of ICD (Bell and Baron, 1959) requires the relatively expensive TPN.

This work is being continued, in collaboration, by Dr. J. C. Edozien at Ibadan, with special reference to serial observations and correlation of these serum enzyme changes with other abnormalities.

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THE BUFFERED PERFORMIC-ACID-ALCIAN-BLUE-PERIODIC-ACID-SCHIFF METHOD FOR THE DIFFERENTIATION OF BASOPHILS IN THE HUMAN AND RAT PITUITARY

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The difficulty of using a single method for staining rat and human pituitary was investigated by testing separately and in different combinations the effects of the various components in the performic-acid-alcian-blue(P.F.A.B.)-periodic-acid-Schiff(P.A.S.) method (Adams and Swettenham, 1958). In this way the possibility of inhibiting or enhancing by one procedure the staining effects of the other could be assessed. The profound influence of fixation on these staining methods was demonstrated by employing the same set of staining methods on tissues fixed in different ways.

The possibility that unbuffered performic acid might exert a considerable solvent action on certain proteins was considered to be related to the difficulty of applying the P.F.A.B.-P.A.S. method to the rat pituitary. Buffered performic acid has far less solvent action and the P.F.A.B.-P.A.S. method, modified by the replacement of buffered for unbuffered performic acid, proved to be successful in the demonstration of two types of basophil cells in both rat and man.

The P.A.S. method introduced by McManus (1946) and Hotchkiss (1948) was applied to the human pituitary by Everson Pearse (1949) and to the rat by Purves and Griesbach (1951). It appears to offer the considerable advantage that its effect depends on a predictable chemical reaction. With this method a mucoprotein component was identified in basophils but not in acidophils and thus a chemical differentiation between these cells was established. A further advantage of this type of method, based on a histochemical reaction, is that its application is not dependent on the use of regressive dyes, the differentiation of which depends on the skill of the operator. Some variability in staining reaction is, however, dependent on the effects of different fixatives (Everson Pearse, 1953a). Purves and Griesbach (1951) reported two types of P.A.S.-positive cell in the rat differing in regional distribution, relationship with blood vessels, and the shape and size of the cell and granules. These cells were stated to react selectively to moderate variations in the level of circulatory sex and thyroid hormones, and were called gonadotrophs and thyrotrophs respectively.

The gonadotrophs were described as coarsely granulated oval cells located mainly at the periphery of the gland close to blood vessels and often containing a conspicuous negative image of the Golgi apparatus. They considered that these cells responded to castration and developed a signet-ring appearance, the coalescent vacuoles displacing the nucleus to one side (Severinghaus, 1938).

A modification of the P.A.S. technique was introduced by Wilson and Ezrin (1954), the aim of which was to differentiate basophils into two types (" P.A.S.-red and P.A.S.-purple ") in the anterior pituitary. This method is greatly dependent on variable counterstaining controlled by the operator; it has enjoyed only temporary popularity and has been superseded by a further modification by Ezrin, Swanson, Humphrey, Dawson, and Wilson (1958).

Following the work of Romeis (1940), who described two types of basophil cells using resorcin-fuchsin with a modified Azan counterstain, Halmi (1950, 1951, 1952) described a method for the differentiation of two types of basophil cells in the rat's pituitary using the

aldehyde-fuchsin method of Gomori (1950) with a modified azan counterstain. Those with an affinity for aldehyde-fuchsin (called beta cells) corresponded with the thyrotrophs of Purves and Griesbach, whilst those demonstrated only by azan (called delta cells) corresponded to the gonadotrophs.

The aldehyde-fuchsin method seems applicable only to the rat's pituitary since neither Fand (1955) nor Everson Pearse (1956) was able to obtain convincing results in human material. As Halmi and Davies (1953) described what appeared to be a partial reversal of results if acidified permanganate was used instead of iodine in the preliminary oxidation with the Halmi technique, the type of oxidation required may be of some importance. Thus the use of performic acid as the oxidizing agent with the aldehyde-fuchsin technique allows the demonstration of two types of basophil cells in the human pituitary (Russell, 1956).

The performic - acid - alçian - blue (P.F.A.B.) method for cystine (Adams and Sloper, 1956) coupled with the P.A.S. technique as a counterstain was applied to the human pituitary by Adams and Swettenham (1958). This has been stated to be unsuccessful in the rat (Adams and Everson Pearse, 1959), but, as will be shown, this is principally due to the failure of P.A.S. to act as an effective counterstain in these circumstances.

Danielli (1947) showed that performic acid had a considerable solvent effect on protein in most of the methods in which it was incorporated, and suggested that buffered performic acid was a greatly improved oxidizing agent in that its solvent effect was less. If Danielli's view can be applied to the rat pituitary, then this diminished solvent action may influence the results of staining by the P.F.A.B.-P.A.S. method. It therefore seemed reasonable to investigate the effects of using buffered performic acid as the oxidizing agent in this method.

The purpose of this work is to attempt to apply the buffered P.F.A.B.-P.A.S. method to both human and rat pituitaries, and to investigate the effects of fixation on the various dyes which are incorporated in this method.

Materials and Methods

The pituitaries from 24 normal male and female rats, weighing 170–300 g., were placed in different fixatives and serial sections 5 μ thick were prepared. The sections were mounted in groups of 25 on five slides and subjected to five staining procedures. The sections were arranged in such a way that adjacent sections were stained by different procedures for the purposes of close comparison.

The pituitaries obtained from 24 patients *post mortem* were also fixed in different ways, cut serially and stained in the same way as the rat sections.

Fixatives.—The following were the fixatives used and the times for fixation:

(1) Cold 4% formaldehyde saline, five hours.
(2) 4% formaldehyde saline, 24 hours.
(3) 4% formaldehyde-sublimate-saline, 24 hours.
(4) 70% alcohol, 24 hours.

Stains.—The following were used:

(1) P.A.S.—orange G.
(2) Buffered performic acid-alcian blue (B.P.F.A.B.).
(3) P.F.A.B.
(4) Aldehyde fuchsin and Halmi.
(5) P.A.S. only.
(6) B.P.F.A.B.-P.A.S.
(7) P.F.A.B.-P.A.S.

Stain 2 was alternated in some cases with stain 6 and stain 3 with stain 7.

Rat Pituitary

Results.—In the assessment of the techniques carried out in this work, attention is paid to these P.A.S.-positive cells, and their differentiation into Halmi's beta and delta types will be observed.

Fixation Effects.—It was found in the experiments in this series that positive P.A.S., P.F.A.B., and buffered P.F.A.B. staining was obtained only after fixation with formalin-containing reagents. Of these, the results for the P.A.S. reaction were best after fixation in cold formaldehyde saline, although the results after fixation in formaldehyde saline at room temperature were satisfactory in that the counterstain did not mask the reaction. The final preparations in this latter group were not as vivid as when the temperature of fixation was low. After formaldehyde-sublimate-saline fixation the positive P.A.S. staining was not quite as strong as with formol saline.

The best results for the buffered and unbuffered P.F.A.B. method were obtained after the use of formaldehyde saline and formaldehyde-sublimate-saline fixation for 24 hours. The results in both buffered and unbuffered P.F.A.B. methods were unreliable after fixation in cold formaldehyde saline.

No staining was obtained with the P.A.S. buffered or unbuffered P.F.A.B. or aldehyde-fuchsin methods after fixation with 70% alcohol.

In this series, counterstaining with orange G and haematoxylin did not obscure the P.A.S. reaction. This and the aldehyde-fuchsin and P.F.A.B. methods all gave consistent results with formaldehyde saline and formaldehyde-sublimate-saline fixation.

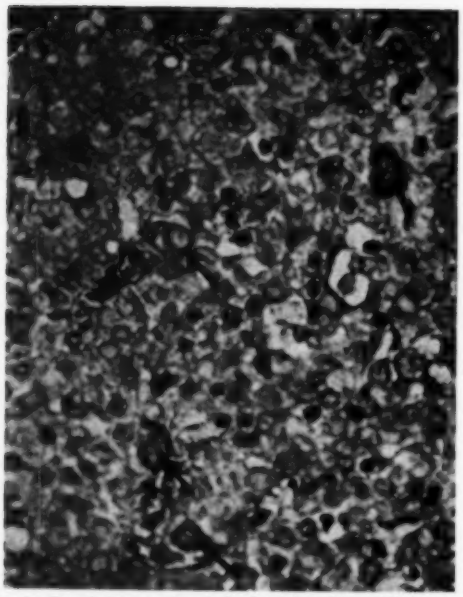

FIG. 1.—Rat pituitary to show lack of staining of basophils (arrows) after pretreatment with unbuffered performic acid. P.A.S. × 540.

Results with P.A.S. Reaction Used as Counterstain to P.F.A.B. Method.—Sections stained by the P.F.A.B. method were counterstained by the P.A.S. reaction (Adams and Swettenham). It was possible to study the effect of the P.F.A.B. method on the demonstration of P.A.S.-positive material. Sections stained in this way were therefore compared with sections stained by P.A.S. alone from material fixed in formaldehyde saline for 24 hours and in formaldehyde-sublimate-saline. This comparison showed that the reaction of material to the P.A.S. technique was much weaker in sections previously stained by the P.F.A.B. method, and cells stained by the P.A.S. technique were not easily seen.

A comparison was then made between sections treated with performic acid and stained by the P.A.S. technique and sections stained by P.A.S. alone, so that the effect of performic acid on the P.A.S. reaction might be assessed. This comparison showed that performic acid alone had the same effect as the P.F.A.B. method on the demonstration of P.A.S.-positive material. The P.A.S.-positive cells were very difficult to see in those sections treated with performic acid before staining with the P.A.S. technique (Figs. 1 and 2).

Comment.—The P.A.S. technique as a counterstain to the P.F.A.B. method appears to have little value in rats when used on material that has been suitably fixed for the demonstration of alcian-blue-positive cells by the P.F.A.B. method.

Use of Buffered Performic Acid.—This problem was investigated by comparing sections stained by performic-acid-P.A.S. with sections stained by buffered performic-acid-P.A.S. and with sections stained by P.A.S. alone. The fixatives for this experiment were formaldehyde saline and formaldehyde-sublimate-saline. This comparison showed that whereas little or no P.A.S.-positive material was demonstrated in sections stained by performic-acid-P.A.S., the demonstration of P.A.S.-positive material in sections stained by buffered performic-acid-P.A.S. compared most favourably with that demonstrated by P.A.S. alone.

Buffered performic acid was therefore used with alcian blue and compared with the P.F.A.B. method, so that the use of buffered performic acid as an oxidizing agent could be investigated and its effect on the demonstration of alcian-blue-positive cells could be noted. This investigation showed buffered performic acid to be as effective

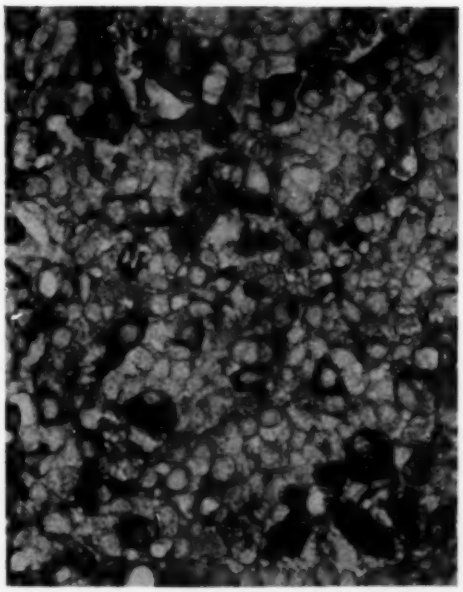

FIG. 2.—Section of same pituitary as in Fig. 1 to show strong reaction in basophils after pretreatment with buffered performic acid. P.A.S. × 540.

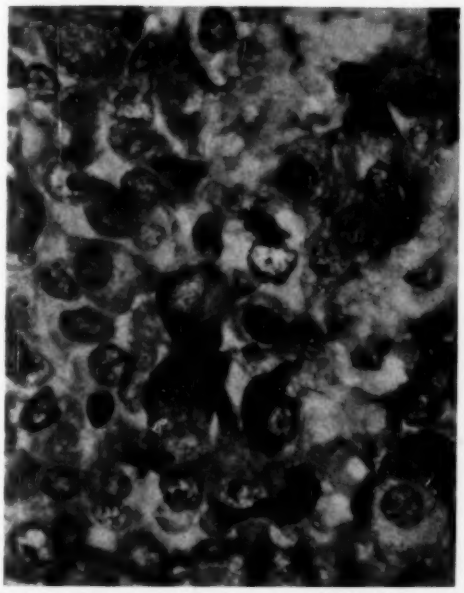

FIG. 3.—Section of rat pituitary to show thyrotrophs at centre stained witn alcian blue. Buffered P.F.A.B.-P.A.S. × 920.

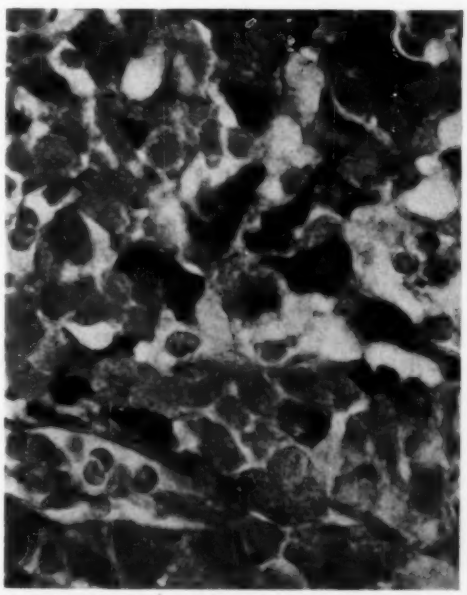

FIG. 4.—Section of same pituitary as in Fig. 3 to show parallel staining of thyrotrophs with aldehyde-fuchsin. Halmi × 920.

as unbuffered performic acid as an oxidizing agent in the performic-acid-alcian-blue method (Figs. 3 and 4).

Comment.—Performic acid and buffered performic acid are of equal value when used with alcian blue for the demonstration of material in certain cells.

The P.A.S. reaction is inhibited after oxidation by unbuffered performic acid but not after oxidation by buffered performic acid.

It is therefore of advantage to use buffered performic-acid-alcian-blue if the P.A.S. reaction is used as a counterstain to the P.F.A.B. method.

With regard to the human pituitary, the effect of both buffered and unbuffered performic acid on the demonstration of P.A.S.-positive mucoprotein in the (S) and (R) cells is identical.

Discussion

With regard to the preservation of the various cell components, it is of interest that P.A.S., P.F.A.B., and buffered P.F.A.B. reactions did not take place after fixation in 70% alcohol, although mucoprotein is said to be precipitated both by alcohol and by acetone (Everson Pearse, 1953b). It is clear that this material was not preserved in these experiments in a physical or chemical state necessary for the development of the P.A.S. reaction.

The present findings do, however, substantiate the view expressed by Everson Pearse (1953b) that formalin is a necessary component in any fixative designed to preserve mucoprotein and to demonstrate its presence subsequently by the P.A.S. reaction. But it should be noted that according to Elftman (1957) the combination of osmium tetroxide at 5° C. with a subsequent chromation in potassium dichromate dissolved in mercuric chloride at 20° C. also apparently preserves P.A.S.-positive material. This particular fixative has not been investigated in this series of experiments. However, of the fixatives which are readily available, easy to manage, and cheap, our experience suggests that, for the combined buffered P.F.A.B.-P.A.S. technique, formaldehyde saline used at room temperature for 24 hours is the most satisfactory method of fixation.

Staining with P.F.A.B.-P.A.S. Combined Method.—The P.F.A.B.-P.A.S. method, which differentiated two types of basophil cell in the human pituitary, failed to do so when applied to the rat pituitary. As it was possible to obtain positive results with the P.A.S. and the P.F.A.B. methods when applied to separate sections the effect of performic acid on the P.A.S. reaction

was investigated and it was found that a satisfactory demonstration of P.A.S.-positive cells could not be obtained after pretreatment with performic acid, unless the performic acid were buffered. The buffered performic acid reagent was therefore incorporated with alcian blue and P.A.S. in the combined method and it was then possible to demonstrate the beta and delta types of basophil cell in rat pituitary material.

The buffered P.F.A.B.-P.A.S. method was also applied to human material and compared with the unbuffered method. It was found to give similar staining results.

The use of performic acid in effecting a differentiation of the basophil cells is paralleled by other procedures. Everson Pearse (1952) lists several techniques by means of which two varieties of basophil may be distinguished. For example, the coupled alkaline tetrazonium reaction with the application of " short-benzoylation " blockade and the Gram stain are two such methods.

The procedure which perhaps resembles the present one to the greatest degree is that described by Wilson and Ezrin (1954). In this method the P.A.S. reaction is developed and the sections first counterstained with orange G, then treated with phosphotungstic acid and finally stained with methyl blue. Certain P.A.S.-positive cells are purple after this procedure; the others remain red. Investigation of this method showed that the differential colour change can be produced by the action of phosphotungstic acid alone on sections stained by the P.A.S. method. This effect could easily be destroyed by washing the section in water, and reproduced by treating again with phosphotungstic acid.

The P.A.S. reaction may be affected therefore by phosphotungstic acid as well as by performic acid in such a way that two varieties of cells with different staining qualities may be differentiated. This does not imply that these effects are necessarily chemically similar, as little is known of either reaction. It also does not imply that the two cell types are necessarily functionally distinct.

The P.F.A.B. technique has frequently been used for demonstrating material relatively rich in cystine (Adams and Sloper, 1956; Adams and Swettenham, 1958; Adams and Everson Pearse, 1959) and the demonstration of centrally distributed basophil cells in the rat pituitary has been held to reflect this property. This distribution of positively reacting material, however, is in conflict with results described by Ladman and Barrnett (1956) using the dihydroxydinaphthyl-disulphide (D.D.D.) method (Barrnett and Seligman, 1952, 1954). Ladman and Barrnett obtained a positive reaction in alpha cells. This discrepancy may be due to a combination of factors. Ladman and Barrnett fixed tissue in 1% trichloracetic acid in 80% alcohol, and it is possible that the mucoprotein in beta cells may not be preserved by this procedure. Thus, as already noted above, alcohol is a particularly poor mucoprotein preservative and Meyer (1945) has claimed that trichloracetic acid is also a poor mucoprotein precipitant. It seems possible that part or all of the beta cell mucoprotein may have been lost. At the same time, D.D.D. is a very sensitive indicator of material containing cystine and a positive reaction may be given by the small quantities almost certainly present in the cytoplasm of alpha cells.

It is a pleasure to record my thanks to Professor D. S. Russell for her help, advice, and encouragement and for the facilities which she made available to me. Thanks are due also to Dr. K. Weinbren for help and criticism in the preparation of the manuscript.

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MISLEADING SPUTUM CARTONS

BY

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Waxed paper sputum cartons obtained from the manufacturers contained very few organisms. Similar cartons which had been stored in hospital wards were often contaminated with hospital dust. This contamination was sometimes sufficiently marked to be misleading. Coliform organisms are particularly liable to cause confusion.

Waxed cartons are useful for collecting sputum, but they must be properly stored.

In many hospitals waxed paper cartons are used for collecting specimens of sputum. Recently we observed substantial deposits of London soot in some unused cartons stored in a ward, and it seemed possible that significant numbers of airborne microbes might also be gaining access to the cartons. A number of unused cartons were therefore sampled to find out whether they contained any organisms, and, if so, whether these were likely to be misleading.

Methods

Source of Cartons.—The cartons were supplied by the manufacturers in batches of 1,000 packed in a cardboard case. The cartons themselves were not individually wrapped, but the lids were wrapped in paper in batches of 20. In the wards the cartons were kept in cupboards, sometimes enclosed in the paper bags in which they were issued by the stores though often exposed to the air in trays or boxes. The lids were usually kept in their original wrappings.

Over a period of three months 100 cartons were collected from the wards and 100 cartons from the stores. Those from the stores included 50 cartons taken at random and 50 obtained from a new case of cartons which we opened ourselves. Lids were placed on the cartons at the time of collection.

Sampling of Cartons.—Digest broth, 2 ml., was added to each carton, and with the lid held on firmly the contents were swirled vigorously 10 times. A sample of the washings (usually 0.5 ml.) was then spread on blood agar plates which had been freshly poured and dried. The plates were inspected after aerobic incubation for 24 hours at 37° C. and re-inspected after a further 24 hours at room temperature. The different types of colony on each plate were noted and representative colonies were identified. The total number of colonies on each plate and the different types of organism which made up this total were counted. The number of organisms in each carton was calculated.

Artificial Contamination of Cartons.—A patient with exfoliative dermatitis, known to disseminate vast numbers of *Staphylococus aureus*, was used as the source of this organism. Large numbers of cartons from a new batch known to contain very few organisms were exposed in his room while the bed was made and the room dusted. From colony counts on blood agar plates exposed at the same time the probable number of organisms falling into each carton was calculated. To prepare cartons containing *Bacterium coli* suitable dust was generated by shaking pieces of cotton cloth which had been impregnated with a broth culture and then dried for 24 hours at 37° C.

Results

Table I summarizes the average counts obtained by sampling 100 cartons from the wards and 100 cartons from the stores. It will be seen that very few organisms were recovered from 50 cartons from a new batch obtained straight from the manufacturers. Higher counts were obtained from 50 cartons collected at random from the stores, suggesting that some contamination occurred in this building. However, these counts were very much smaller than counts obtained from cartons from the wards.

TABLE I

AVERAGE NUMBER OF ORGANISMS ISOLATED FROM UNUSED SPUTUM CARTON

Source of Cartons	No. Sampled	Average No. of Organisms per Carton
Medical and surgical wards ..	100	419
Central stores		
(*a*) At random	50	44
(*b*) New batch	50	3

The counts of total organisms from individual cartons varied greatly and are analysed further in Table II. It will be noted that 70% of the cartons obtained from the stores were sterile compared with only 4% of those obtained from the wards. At the other end of the scale no carton from the stores contained more than 1,000 organisms, but six cartons from the wards gave counts higher than this.

TABLE II

DISTRIBUTION OF COUNTS OF TOTAL ORGANISMS

	Total No. of Organisms per Carton					
	Sterile	1–10	11–100	101–1,000	1,001–10,000	>10,000
No. of cartons in each category from:						
Wards ..	4	11	44	35	5	1
Stores ..	70	15	9	6	0	0

The flora of contaminated cartons usually consisted of several different types of organism. To indicate the types most frequently encountered a record was made of the number of cartons which were shown by differential counts to contain more than 100 organisms of the same type. Of the 200 cartons sampled, two contained more than 100 of each of three different types of organisms, eight contained more than 100 of two different types, and 26 contained more than 100 of only a single type. The predominant organisms isolated from these 36 cartons are shown in Table III.

TABLE III

NUMBER OF CARTONS CONTAINING MORE THAN 100 ORGANISMS OF THE SAME TYPE

Type of Organism	Cartons from Wards			Cartons from Stores		
	101–500	501–1,000	>1,000	101–500	501–1,000	>1,000
	Organisms/Carton			Organisms/Carton		
Staph. aureus ..	4	0	1	0	0	0
Other staphylococci ..	15	1	2	3	0	0
Bact. coli ..	1	0	1	0	0	0
Other coliforms	1	1	1	0	0	0
Streptococcus spp.	3	1	0	1	0	0
Neisseria spp. ..	1	0	0	2	1	0
Bacillus spp. ..	2	0	1	1	0	0
Moulds ..	3	1	0	0	0	0

The organisms most commonly encountered were staphylococci. Most of these were coagulase-negative strains variously considered as belonging to the genera *Micrococcus*, *Sarcina*, and *Staphylococcus*, but five cartons from the wards contained more than 100 coagulase-positive *Staph. aureus*, one particular carton containing no less than 2,400 *Staph. aureus* out of a total flora of 5,400 organisms.

Two cartons contained more than 100 typical *Bact. coli*. One of these cartons was the most heavily contaminated in the whole series. Out of a total flora conservatively estimated at 14,000 organisms, 12,400 were *Bact. coli* and, since many of the remaining organisms were *Streptococcus faecalis*, it seemed likely that this carton had been contaminated with faeces. The other coliform organisms listed in Table III were strains of *Achromobacter* isolated from two cartons and paracolons isolated from a single carton.

Five cartons contained more than 100 streptococci: in two cartons these were of "viridans" type, in two they were non-haemolytic, and in one they were *Str. faecalis*.

Other organisms found in large numbers were Gram-negative cocci, aerobic spore-bearing bacilli, and moulds. With one exception these all belonged to non-pathogenic species: the exception was that one carton gave an almost pure growth totalling 360 colonies of *Aspergillus fumigatus*.

An important organism not shown in Table III is *Proteus*. Sampling plates from three cartons obtained from the wards were overgrown with this organism. No *Proteus* was isolated from cartons obtained from the stores. From cartons obtained from the wards *Pseudomonas pyocyanea*, a member of the Friedländer-aerogenes group and a β-haemolytic streptococcus (which did not belong to Lancefield's Groups A, B, C, D, or G) were isolated from one carton each in very small numbers (less than 10 per carton).

The extent to which cartons were contaminated bore no obvious relation to the type of ward or their rate of turnover of cartons. In general the total counts were lowest in those wards, medical or surgical, where cartons were stored under conditions preventing free access of dust.

Support for the view that contamination of the cartons was usually airborne was given by the fact that the incidence of *Staph. aureus* in the flora of cartons agreed surprisingly well with what was already known of the incidence of this organism in the air of different wards. In particular, the air of two wards used for patients with skin diseases was usually far more heavily polluted with *Staph. aureus* than the air in any other ward in the hospital; these two skin wards were the main source of *Staph. aureus* from sputum cartons. The numbers of these organisms isolated from the two skin wards and 18 other wards are compared in Table IV. Of the nine cartons from the skin wards which grew *Staph. aureus*, four contained

TABLE IV

RECOVERY OF *STAPH. AUREUS* FROM ALL SOURCES

	Source of Cartons	
	Skin Wards	Other Wards
No. of cartons sampled	10	90
,, ,, ,, yielding *Staph. aureus*	9	20
Total no. of organisms isolated ..	7,388	34,504
,, ,, ,, *Staph aureus* ,, ..	2,864	478
Staph. aureus expressed as % of total organisms	38·8	1·4

No *Staph. aureus* were isolated from 100 cartons from the stores.

TABLE V

RESULTS OF CULTURING SPECIMENS FROM CONTAMINATED CARTONS

	Sputum	Saliva
Specimens yielding *Staph. aureus* after collection in:		
(*a*) Clean cartons	0/24	0/12
(*b*) Cartons containing 100–200 *Staph. aureus*	2/24	0/12
Specimens yielding *Bact coli* after collection in:		
(*a*) Clean cartons	0/12	2/12
(*b*) Cartons containing 100–200 *Bact. coli*	4/12	10/12

more than 100 of these organisms compared with only one of the 20 cartons from the other wards. No *Staph. aureus* was isolated from 100 cartons obtained from the stores.

Of the various organisms found in contaminated cartons it was thought that *Staph. aureus* and *Bact. coli* would be most likely to cause confusion in the examination of specimens of sputum. Experiments were therefore carried out with cartons which had been deliberately contaminated with dust containing these organisms.

Specimens of sputum arriving in the laboratory were cultured in the routine way and if no *Staph. aureus* or *Bact. coli* were found the specimens were divided into two portions: one portion was placed in a carton obtained from a fresh batch known to contain few or no organisms; the other portion was placed in a carton contaminated with 100–200 *Staph. aureus* or *Bact. coli*. The two cartons were then handed as unknowns to student technicians who were asked to culture the specimens on blood agar and report on the presence or absence of these two organisms. A similar procedure was used for specimens of saliva. The results of some of these investigations are shown in Table V.

In these and other experiments it was difficult to detect small numbers of *Staph. aureus*. *Bact. coli*, on the other hand, was recognized much more often since, unlike *Staph. aureus*, even a single colony of *Bact. coli* stands out in the area of heaviest inoculum. With specimens of saliva—and in this laboratory some 20% of sputum specimens consist of little else—small numbers of *Bact. coli* were recognized very easily. In normal circumstances an occasional colony of a coliform organism would be ignored. However, with three specimens of saliva and one specimen of sputum more than 10 colonies of *Bact. coli* were counted, and it is probable that in the ordinary way these organisms would have been investigated further.

Discussion

To assess the significance of organisms isolated from sputum is one of the most difficult problems in routine clinical bacteriology. In recent years the problem has become more difficult because the widespread use of powerful antibiotics has vastly increased the number of specimens containing *Bact. coli*, *Proteus*, paracolons, and other Gram-negative bacilli which are able to gain a footing in the respiratory tract when the normal coccal flora are suppressed. Although these coliform organisms are often present in only small numbers there are advantages in investigating them, as the findings may occasionally be of help if the same organisms are isolated from subsequent specimens from the same patient.

If attention is going to be paid to small numbers of coliform organisms it is reasonable to insist that the specimen should be collected in a clean container. Of all the organisms isolated from contaminated cartons it seems that coliform organisms are most likely to cause confusion. How often this occurs is largely irrelevant as the problem can be solved quite simply: sputum cartons should be individually wrapped, kept in a dust-proof container, or stored with their lids on.

J. clin. Path. (1960), 13, 264.

TECHNICAL METHODS

A Survival Technique for Obtaining Large Volumes of Blood from Rodents

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(RECEIVED FOR PUBLICATION OCTOBER 24, 1959)

In survival experiments rodent blood is classically obtained from the tail or iliac veins. Only small samples of blood, particularly in the case of mice, can usually be obtained from techniques involving the use of either of these routes, and moreover these samples often exhibit some haemolysis. The object of this communication is to present a simple technique by which these difficulties may be obviated.

Technique

Mice.—The animal is anaesthetized with ether and ethyl chloride and is then placed on its back on a cork board. An assistant extends the neck by retracting the lower jaw with forceps, at the same time holding back the front paws with the thumb and first index finger (Fig. 1). A transverse incision, 5 mm. in length, is made lateral to the midline of the neck in the course of the internal jugular vein (Fig. 1). This vein is large, easily identifiable, and can be punctured with a No. 16 needle attached to a syringe. Care must be taken to prevent the needle from tearing the friable wall of the vein. Blood is then aspirated in stages, allowing the vein to fill up between each application of suction, and in this way 1 ml. or more of blood can easily be removed. Slight pressure with a cotton-wool plug is applied for a few seconds after the needle is withdrawn. One skin suture is then inserted. The whole procedure, from the start of anaesthesia to recovery of consciousness, takes less than two minutes.

Rats.—The operative procedure for rats is similar to that for mice, except that they are anaesthetized with ether alone and subsequently positioned on a hollowed wooden board and the neck extended by catching the top molars on a metal wire across the board. Five millilitres of blood can then be removed through a No. 1 needle from these animals. During removal of blood no further anaesthetic should be administered, as fatalities may occur at this stage with too deep anaesthesia. The animals should be returning to consciousness by the time the suture is applied.

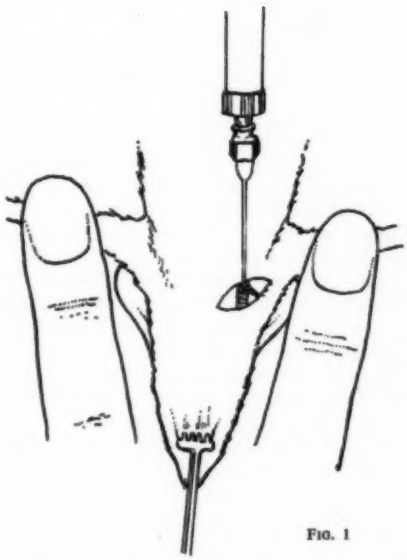

FIG. 1

Discussion

The technique described above is simple and can be easily mastered after a little experience. Two hundred consecutive blood samples were withdrawn from rats and mice in this way. These particular samples of blood were collected into siliconized tubes and centrifuged within one hour. Freedom from haemolysis was demonstrated spectroscopically in 98% of these samples. No animal died as a result of the operation or suffered any ill effects. On some occasions blood was taken on successive days.

Summary

A simple survival venepuncture technique by which large volumes of non-haemolysed blood can be taken from rats and mice is described and evaluated.

We are extremely grateful to Mr. G. Lyth for the illustration in this paper, and to Dr. S. S. Epstein for his co-operation.

A Rapid Method of Bleeding Mice

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(RECEIVED FOR PUBLICATION DECEMBER 23, 1959)

Some laboratories where mice are used as experimental animals find difficulty in obtaining blood from these animals in volumes sufficient for micro or semi-micro analysis. A method has been used for some years in these laboratories which enables as much as 0.75 ml. of whole blood to be collected per bleeding without any adverse effect, thus enabling the animals to be bled at repeated intervals. In response to numerous requests, the method is described here in some detail.

Experimental

The method of bleeding was adapted from one devised by Bullen and Batty (1956).

Mice are placed in a heating box (Fig. 1) and their body temperature raised, with the aid of two 100-watt globes for at least five minutes, to distend the tail veins. It has been found that coloured mice do not tolerate heat treatment as well as albino mice and therefore caution is urged in heating coloured mice. The mice are then anaesthetized and the tip of the tail, about 1 cm. for mice not previously bled, and 2 or 3 mm. for others, amputated with a pair of sharp scissors. The stump of the tail is then quickly lowered into a labelled Wassermann tube contained in a large tube under a negative pressure of 10 cm. Hg (Fig. 2).

The degree of anaesthesia should be sufficient to prevent the mouse struggling during the time it is placed over the larger tube. It is important that a seal be formed by the mouse's body and the mouth of the large tube so that a constant negative pressure is maintained. Negative pressure in excess of 10 cm. Hg should be avoided, as this often causes faecal matter and urine to be excreted into the Wassermann or sample tube.

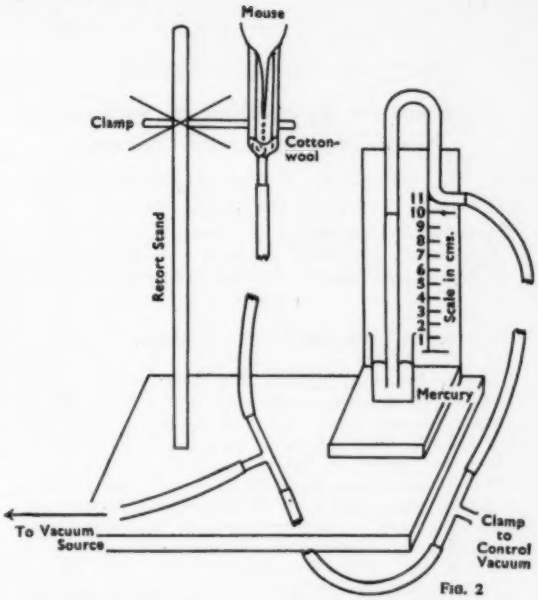

FIG. 2

Blood clots are freed from the side of the tubes by means of a finely drawn solid glass rod and the tubes allowed to stand overnight at 21° C. The serum is pipetted into clean glass tubes, centrifuged to remove cells, and stored at −20° C.

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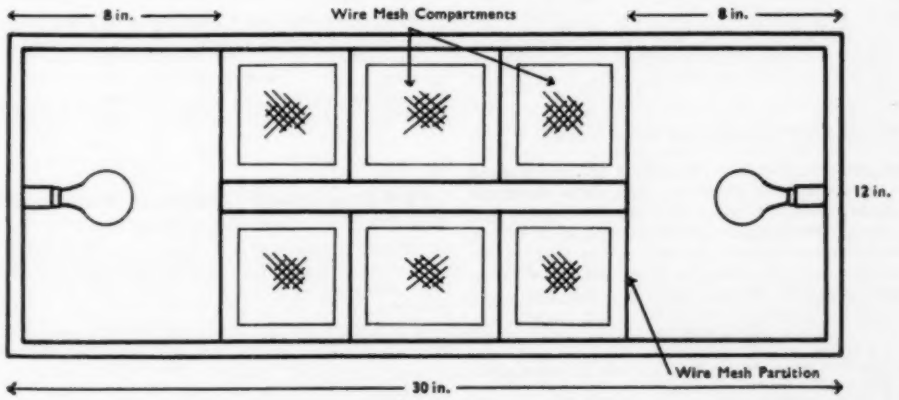

FIG. 1

X

A Method for the Simultaneous Separation of Human Thrombocytes and Leucocytes

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(RECEIVED FOR PUBLICATION DECEMBER 12, 1959)

The following method has been evolved from techniques described previously for the separation of thrombocytes (Gurevitch and Nelken, 1954) and leucocytes (Walford, Peterson, and Doyle, 1957). It has been tested on 200 various blood samples.

Only siliconized glassware was used. All centrifugations were carried out in a refrigerated centrifuge at 4° C. A 1% solution each of Na_2 sequestrene (disodium ethylene diamine tetra-acetic acid) and triton W.R. 1339 (oxyethylated tert. octyl phenol formaldehyde polymer) in 0.7% sodium chloride solution served as the anticoagulant.

Blood was withdrawn into a siliconized syringe containing anticoagulant in the proportion of 1:9 and divided into 5 aliquots of 20 ml. in 50 ml. round-bottomed centrifuge tubes. The blood was then centrifuged in a horizontal centrifuge at 800 r.p.m. (110 *g*) for seven minutes, causing the sedimentation of erythrocytes and leucocytes. The thrombocyte-rich plasma supernatant was drawn off and recentrifuged at 2,000 r.p.m. (600 *g*) for a further seven minutes. This second sediment contained the thrombocytes.

The thrombocyte-poor plasma supernatant was returned to the tube containing the erythrocytes and leucocytes and mixed well with the sedimented cells. To this "thrombocyte-poor blood" ¼ volume of a 6% dextran solution in physiological saline (intrinsic viscosity 0.40/20° C.) was added. The dextran was first warmed to 37° C. and mixed with the blood by transferring the dextran-blood mixture from one 50 ml. siliconized tube to another. The mixture was allowed to stand at room temperature (20°–25° C.) for 30 minutes at an angle of 45°.

During this time sedimentation of the erythrocytes took place. The supernatant plasma containing the leucocytes, some erythrocytes, and few thrombocytes was centrifuged at 600 r.p.m. (60 *g*) for seven minutes. To the sediment thus obtained 6 ml. of distilled water was added to haemolyse the remaining erythrocytes and the tube shaken for exactly 25 seconds. Then 2 ml. of a 3.4% NaCl solution was added in order to restore isotonicity. The tube was again centrifuged at 600 r.p.m. (60 *g*) for seven minutes. The haemolysed supernatant was removed, and the sediment contained the white blood cells.

The thrombocytes and leucocytes obtained in this manner could be diluted with normal saline as desired. The cells could be washed repeatedly in a washing fluid made of the standard anticoagulant solution diluted 1:10 with normal saline (unbuffered *p*H 6–7) without clumping. The average thrombocyte yield was 60%. The contamination of the preparation by red blood cells was one erythrocyte to every 2,000 thrombocytes, and one leucocyte to 5,000–10,000 thrombocytes. The average leucocyte yield was 65% and the contamination by thrombocytes was 2–3 thrombocyte ghosts to every 100 leucocytes and 1–2 erythrocyte ghosts to 100 leucocytes. All counts were performed with the phase-contrast microscope.

The method described above did not present any difficulties, except in a few cases of leukaemia and in severe cases of polycythaemia. In the latter, the addition of ¼ volume of normal saline to the whole blood helped to improve the results. The rapid separation of thrombocytes and leucocytes, both of which are obtained in high yields, is a great advantage of this method. Furthermore, the cells appear morphologically intact, and have been used in immunological, metabolic, coagulation, and phagocytosis studies with good and reliable results.

This study has been assisted by a grant from the Leukaemia Research Foundation, Chicago, Ill., U.S.A.

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A Rapid Method of Bleeding Mice (contd.)

Hundreds of mice have been bled by this method in the determination of haemolytic titres to sheep red cells (Sobey and Adams, 1955; Sobey, Adams, and Claringbold, 1956; Claringbold, Sobey, and Adams, 1957) without mortality. Mice may be bled at the rate of one every one and a half minutes with an average yield of 0.25 ml. serum per bleeding.

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First International Congress of Histochemistry and Cytochemistry

The First International Congress of Histochemistry and Cytochemistry will be held in Paris from August 28 to September 3, 1960.

Nomogram for the Computation of the Corrected Colour Value in 17-Ketosteroid Estimation by the Zimmerman Reaction

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(RECEIVED FOR PUBLICATION AUGUST 28, 1959)

The M.R.C. committee on clinical endocrinology (1951) recommends correcting the green extinction value in the determination of 17-ketosteroids by the formula:

$$\text{Corrected green extinction value} = \frac{\text{observed green extinction value} - 0.6 \times \text{observed violet extinction value}}{0.73}$$

This formula, derived largely from the work of Talbot, Berman, and MacLachlan (1942), can easily be converted into the form $x=m_1y+m_2z$ which allows the construction of a simple nomogram (Feldman, 1935). The latter greatly accelerates calculation when several estimations are being done together, and reduces the risk of arithmetical error.

Thus if the observed extinction at 430 mμ $=E_{430}=x$,
the observed extinction at 520 mμ $=E_{520}=y$,
and the corrected extinction $=E_{(corr.)}=z$,
the formula given above can be written:

$$y=0.73z+0.6x \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

To construct a nomogram of sufficient accuracy take a large sheet of 1 in. graph paper (20 in. by 15 in.), scaled in 1/10 inches, and draw three parallel vertical lines, 15 in. long and 5 in. apart and a base line along the bottom as shown in Fig. 1, and label the vertical lines X, Y, and Z, from left to right. On X will be plotted the observed E_{430} and on Y the observed E_{520}, and Z will then give $E_{(corr.)}$. The line X is marked off in 2 in. divisions, each to correspond to 0.1 unit. It is subdivided further and at the point 0.730 unit a line is drawn parallel to the base to cut Z and this point is marked 0.600 unit and the length between the point and the base is divided into six equal divisions and subdivided further and also extended a little way upwards as shown.

In order to graduate line Y, tie-lines are placed between point 0.300 on X and 0.301 on Z and where Y is cut corresponds to 0.400 unit on this scale. Similarly by joining point 0.600 on X and 0.603 on Z the point 0.800 is found. Confirmation of the above points can be obtained by joining 0.500 on X with 0.137 on Z and 0.700 on X with 0.521 on Z, after which Y can be divided into eight equal divisions and further subdivided. The main X, Y, and Z lines and their divisions are best traced on to a sheet of white Bristol board by pricking through the marks with a sharp needle after which the nomogram is drawn on the cardboard in Indian ink.

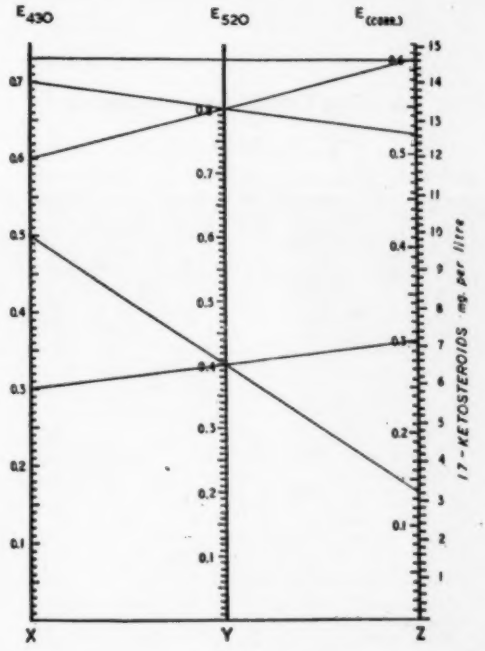

FIG. 1

To evaluate the corrected reading the observed reading at 430 mμ is joined with that obtained at 520 mμ, using a thin transparent ruler, and the required result is read off on line Z.

When a standard calibration for 17-ketosteroids determination has been made, a separate graph is plotted for the corrected reading against dehydroepiandrosterone expressed in terms of milligrams per litre of urine. The resultant line may not be strictly linear, but the interpolated values can be transferred to the nomogram and plotted alongside the corrected values on line Z, preferably in pencil to allow for periodic revision, so that a direct interpolation of 17-ketosteroids can be obtained. The nomogram shown above illustrates the inclusion of such a scale, which is shown to the right of line Z, and has been obtained using dehydroepiandrosterone as the standard substance and is based on the assumption that the residues from 100 ml. samples of urine have been taken up in 2 ml. ethanol before the colour development stage in the M.R.C. method (1951).

To obtain the urinary 17-ketosteroids in milligrams per 24 hours, the only calculation necessary is the multiplication of the result obtained from the nomogram by the 24-hour volume in litres.

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A Micro Ultrafiltration Apparatus

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(RECEIVED FOR PUBLICATION FEBRUARY 26, 1960)

It is a great advantage to be able to obtain protein-free ultrafiltrate in a simple manner from plasma or other body fluids for micro analysis. Dilution of the substance to be analysed is avoided. Errors due to dilution, protein precipitation, and transfer are minimized. Ultrafiltration on a micro scale is used in this laboratory also for paper chromatography of sugars, plasma calcium fractions, and for body space determinations. Plasma ultrafiltrable calcium levels using this apparatus have been compared with the clinical picture in neonatal tetany by Craig (1958) and by Craig and Buchanan (1958). The estimation of plasma water and the calculation of a Donnan equilibrium in body space determinations are avoided when ultrafiltrate is used.

Two methods are available for 3–5 ml. volumes of plasma. In the first method the fluid is driven through a membrane (which is impermeable to protein) by centrifuging (Toribara, Terepka, and Dewey, 1957). Using a laboratory centrifuge, kept at 37° C., these workers obtained 0.5 ml. of ultrafiltrate from 3 ml. of plasma in three hours. This fraction has been found to decrease as the method is scaled down to volumes of 0.2 to 1.0 ml., since losses play a greater part. In the second group of methods the ultrafiltrate is obtained by maintaining a pressure differential across a membrane. Where *p*H and gas tensions are important, e.g., in measuring the ultrafiltrable calcium levels in plasma, it is preferable to use the positive pressure of a column of mercury on the plasma side of the membrane. Lavietes (1937) designed an apparatus for this purpose. However, 3–5 ml. of plasma was necessary. The two glass hemispheres were fragile and they were difficult to clamp into position. The following apparatus has been designed for 0.2 to 1.0 ml. volumes of plasma or blood. These volumes can be obtained with ease by skin puncture in infants and children (Wilkinson, 1960). The apparatus has been in use for five years satisfactorily. The actual filling of the apparatus is very simple and takes two minutes. Filtration proceeds overnight without attention at any desired temperature. At 20° C. and 37° C., 0.5 ml. of ultrafiltrate, and at 4° C., 0.3 ml. of ultrafiltrate, was obtained from 1.0 ml. of plasma in eight hours through a membrane of 80 mm.^{2} area using a pressure of 760 mm. of mercury.

Construction

The glass parts are blown from two flanged tubes (10 mm. bore, 30 mm. flange diameter) and a diagonal-bore spring-loaded tap. The approximate dimensions are indicated in Figs. 1 and 2. The clamp is made from brass rod and strip (Fig. 3). The membrane is cellophane P.T. 600.* The mercury is purified carefully in the normal way, but traces of calcium must be removed with strongly alkaline E.D.T.A. when determining ultrafiltrable calcium levels in plasma. The complete apparatus consists of a glass mercury reservoir connected by elastic P.V.C. tubing to the filtration chamber. A screw tunnel clamp is placed on the tubing near to this. The cellophane membrane is held securely between the glass flanges which are compressed by the brass clamp. The flanges are protected by thick rubber collars.

(1) **Plasma Ultrafiltrate Technique for Calcium.**—Blood obtained by skin or vein puncture is delivered below liquid paraffin with the minimum of contact with air. Heparin is used as an anticoagulant. The tube is capped and centrifuged. The plasma is removed from the cells and stored in a refrigerator for not longer than 24 hours at −10° C. The glass apparatus is cleaned in strong alcoholic potassium hydroxide, washed with glass-distilled water, and dried in an oven. The tap and opposing surfaces of the flanges are smeared lightly with high vacuum silicone grease. A small square of cellophane which has been soaked in distilled water for one to three hours is blotted carefully between ashless filter paper and placed over the flange and opening of the lower compartment. The upper compartment is placed gently over this, flange to flange, and the apparatus is fitted into the brass clamp. The nuts are tightened so that the plates compress the flanges firmly and equally. The plastic tubing is attached to the side tube of the apparatus, and the funnel and tubing are filled with mercury. The brass clamp is rotated so that the glass outlet tube points upwards and the tunnel clamp in the plastic tubing is opened. The glass tap is opened gently and mercury is allowed to fill the compartment and the capillary outlet tube to its tip. The tap is closed and the clamp rotated so that the outlet tube once more points downwards. Mercury is placed in the upper glass compartment to a depth of 2 to 3 mm. to facilitate removal of the ultrafiltrate. The upper opening of the compartment is closed with a rubber policeman. The plasma is equilibrated for three minutes with "alveolar" air which has been passed over wet glass beads. The small tube containing the plasma is arranged so that the outlet tube of the apparatus passes to the bottom of the tube. The mercury funnel is lowered below the level of the apparatus and the glass tap is opened gently so that plasma is drawn up into the lower compartment. The tap is closed and the mercury funnel is raised to a height of 76 cm. above the apparatus. The glass tap is opened gently to expel the plasma in the

*British Cellophane Ltd., 9, Henrietta Place, London, W.1.

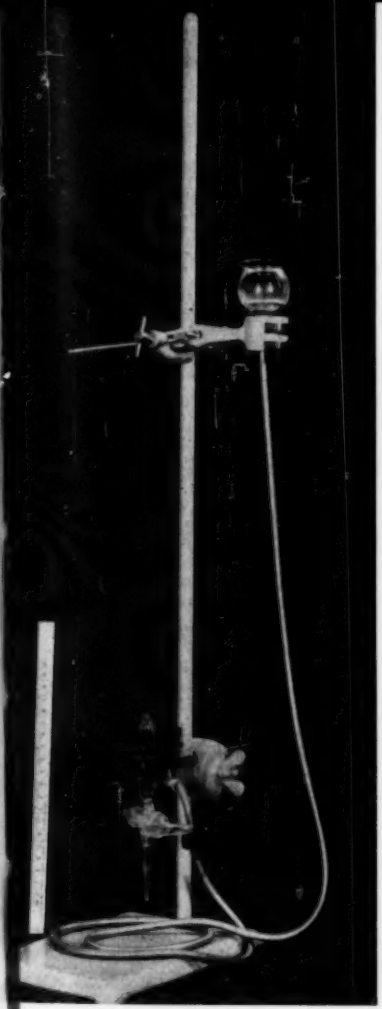


FIG. 1.—The complete apparatus.

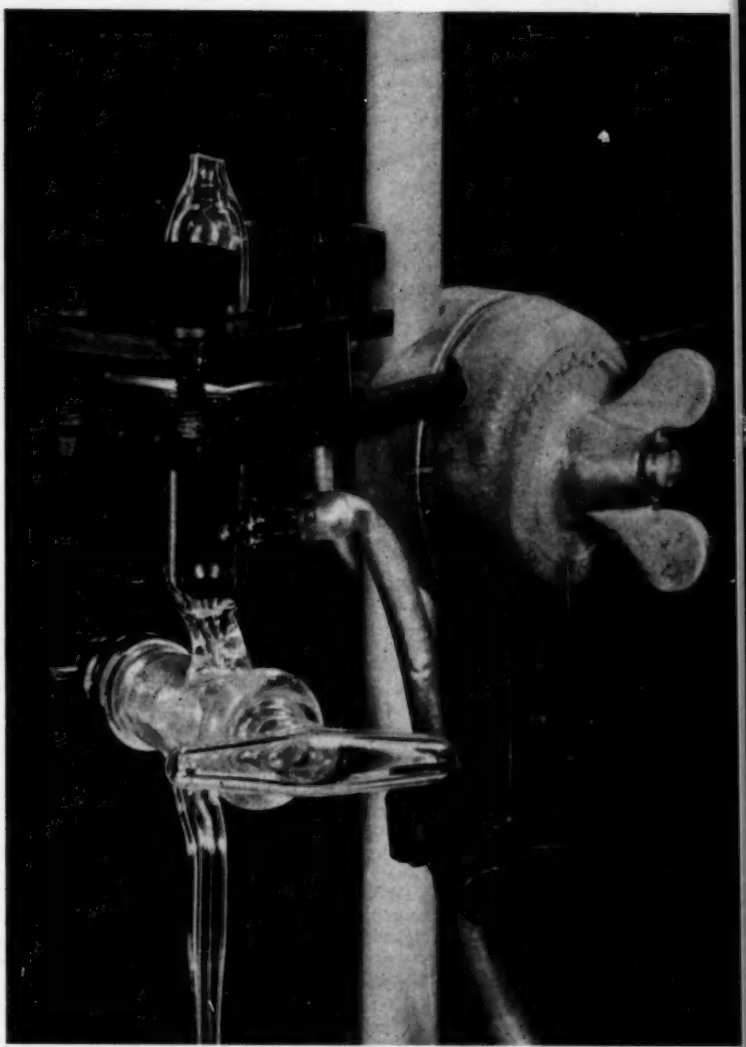
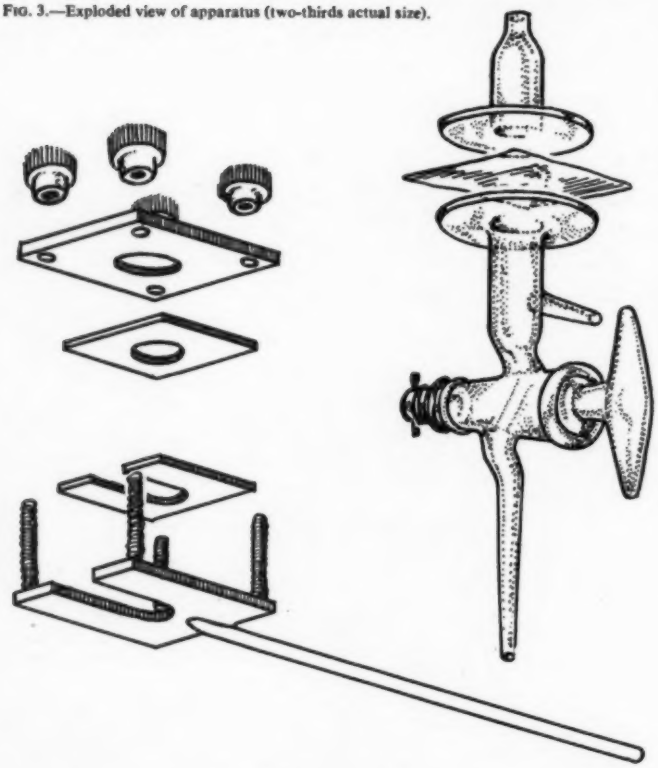


FIG. 2.—The glass filtration apparatus and brass clamp (actual size).

FIG. 3.—Exploded view of apparatus (two-thirds actual size).



capillary tube back into the specimen tube. The tap is then closed again. When using 1.0 ml. of plasma, 760 mm. Hg pressure, and a membrane of 80 mm.^{2} area, ultrafiltration at 20° C. and 37° C. proceeds approximately linearly until 0.5 ml. of filtrate is obtained in eight hours. A total of 0.65 to 0.7 ml. is obtained in 15 hours (overnight). At 0° C. ultrafiltration proceeds linearly for 12 hours until 0.5 ml. of ultrafiltrate is obtained and 0.65 to 0.7 ml. requires 24 hours. It is convenient to allow ultrafiltration to proceed overnight. The ultrafiltrate is removed from the upper compartment by means of a fine Pasteur pipette and may be analysed for calcium (or magnesium) using 0.2 ml. (Wilkinson, 1957). The *p*H of the ultrafiltrate is checked (after equilibration) with a B.D.H. capillator (phenol red). Protein is tested for with sulphosalicylic acid.

(2) **Ultrafiltration Not Needing pH Control.**—Ultrafiltrate may be prepared for the chromatography of sugars, amino-acids, organic acids, 'c.; for determining body space volumes, and in general for micro analyses where the concentration of a substance must be kept as high as possible. Heparinized blood is collected and centrifuged in the usual manner. The apparatus is assembled and filled with mercury. Mercury is placed on the upper surface of the membrane. Whole blood (preserved with fluoride) has been ultrafiltered, the ultrafiltrate desalted with an ion exchange resin, and the sugars present identified and their concentration measured approximately by paper chromatography. Other plasma substances, whose concentration is not affected by prolonged contact with red cells, may be estimated after ultrafiltration of whole blood. After filtration, the clear fluid may be measured with a constriction pipette, and frequently a colorimetric procedure can be carried out directly in a single tube or cuvette. This makes a significant contribution to the accuracy of a method since errors inherent in plasma protein precipitation and transfer pipettings are avoided.

Summary

An apparatus is described for ultrafiltering volumes of 0.2 to 1.0 ml. of body fluids under strictly anaerobic conditions or with *p*H control if necessary. The operation is simple, dependable, and has been in routine use for five years.

I should like to thank the Department of Medical Illustration for Figs. 1, 2, and 3; Messrs. Laboratory Glassblowers Co. Ltd., Valley Works, Lane End Road, Sands, High Wycombe, for supplying the glass parts to my design so ably; and D. J. Perry & Co. Ltd., 7, Avon Trading Estate, Avonmore Road, London, W.14, for supplying the grade of cellophane.

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THE ASSOCIATION OF CLINICAL PATHOLOGISTS

The 64th general meeting was held at the University College of South Wales and Monmouthshire, Cardiff, on April 7, 8, and 9, 1960.

Abstracts of papers given follow.

Effect of Adrenalin Infusion on the Plasma Concentrations of Antihaemophilic Globulin in Normal Subjects and Haemophiliacs

G. I. C. INGRAM (St. Thomas's Hospital, London) said that experiences in two haemophiliacs suggested that acute, severe bleeding was associated with a sudden rise in plasma antihaemophilic globulin (A.H.G.) activity. This seemed analogous to a previous observation that the clotting time in silicone-coated tubes shortened markedly after adrenalin infusions in normal subjects; and the effect of adrenalin on plasma A.H.G. activity was therefore investigated. Adrenalin (10–15 μg./min.), isoprenalin, (3–5 μg./min.), and *nor*adrenalin (10–15 μg./min.) were infused for 10 min. in six normal subjects; so far, on the average, A.H.G. activity had risen after each drug, but most with adrenalin, with considerable individual variation. In two haemophiliacs, a marked rise was noted in one patient after two adrenalin infusions, but a fall in the other patient; in two severe haemophiliacs, there was no rise.

Other experiments suggested that the effect is not an artifact related to contact factors or to fibrinolytic activity, and does not appear to be related to a fall in the eosinophil count; a suggestive correlation has been observed between the A.H.G. rise and the rise in blood glucose concentration.

Listerian Control of Staphylococcal Hospital Cross-infection

S. D. ELEK and P. C. FLEMING (St. George's Hospital, London) said that Lister's carbolic spray was replaced by aseptic surgery, but staphylococcal wound infection is still a problem. The main reservoir of staphylococci is believed to be the nose, from which dust, fresh individuals, and wounds are infected. The risk of wound infection in hospitals is related to the degree of pollution of the environment.

Gould showed that in hospitals there is a detectable amount of penicillin floating in the air, leading to a selective exclusion of penicillin-sensitive staphylococci from the noses of personnel. The authors made a deliberate use of this observation to eliminate nasal carriage. A new experimental antibiotic, AB 1241, was selected as it fulfilled the following requirements: (1) It was active against all of 400 strains of *Staph. pyogenes* tested. (2) It was active in concentrations of 1 to 5 μg. per ml. and highly bactericidal at about the bacteriostatic level. (3) Resistance could not be induced in 12 strains by 20 serial passages through Szybalski gradient plates. The substance is odourless and non-irritant.

A maternity unit was chosen for the spraying experiment because of the known high incidence of nasal carriage in infants. Approximately 55% of daily nasal swabs taken from each of 58 newborn infants during their first 10 days of life grew pyogenic staphylococci. Daily spraying of 1 g. of the drug was next carried out in one ward and nursery occupying about 15,000 cu. ft., using a commercial window-cleaning sprayer. This halved the nasal carriage but failed to abolish it. Increasing the concentration to 1 g. four times a day led to further reduction for the next 20 babies. Thereafter a cumulative effect became manifest as the drug began to build up in the environment. No pyogenic staphylococci at all were isolated from the next 24 babies during their 10-day stay in hospital. No resistant strains emerged, and no hypersensitivity phenomena have been observed over the 20-week period. This appears to be a simple and effective means of eliminating nasal carriage in babies, and is put forward as a possible approach for the control of wound sepsis in hospitals.

The Nature of Vaginal Discharge

J. A. BOYCOTT (Taunton) reported that, of 561 specimens of vaginal discharge from women between the ages of 15 and 50, 418 contained little or no pus. The reaction, cytology, and flora of this non-purulent discharge make it probable that it is an exaggerated flow of the normal secretions of the vagina and cervix. One hundred and thirty-four out of 143 purulent discharges contained *Trichomonas spp.* with a mixed and inconstant bacterial flora. Nine purulent discharges appeared to be due to bacterial infection.

Candida spp. were found in one purulent and in 64 non-purulent discharges. It is suggested that these yeasts are not parasitic upon the vaginal epithelium but derive their nutrition from the secretions of the epithelium.

In children below 15 and in women over 50 the picture is different. The discharge in 14 out of 16 children and in 16 out of 18 older women appeared to be due to bacterial infection.

Cross-infection in Maternity Hospitals

BERYL D. CORNER and WILLIAM A. GILLESPIE (Bristol) also spoke of cross-infection in maternity hospitals, and showed that the spread of staphylococcal infection was quite acute once there was an accumulation of resistant strains. They found that the ordinary accepted methods of interruption of spread were disappointing in the long run, and that the infection arose from adult carriers and was spread superficially by nose and skin, particularly the umbilicus. By the continuous use of hexachlorophane powder and by the instillation of hibitane and neomycin in the noses of mothers and babies there was

a marked reduction in the staphylococcal rate in both mothers and babies, particularly in the follow-up clinics in the incidence of breast abscesses and sticky eyes in the children. A word of warning was, however, sounded in that it was evident in the most recent survey that *Monilia* were now taking a firmer hold in the wards, and in the last bacterial survey there were 25 cases of spots due to staphylococci, three streptococcal abscesses, and 72 cases of monilial infection. When asked whether the nose was still the main source of infection, they felt that as regards the babies the umbilicus was probably more important, as it became colonized rather earlier.

Hospital Vacuum Cleaner as Subject of Laboratory Study

J. G. BATE (Princess Beatrice Hospital, London) said that to dispel uncertainty about the safety of suction cleaners in hospitals wards, the bacterial content of the air from 10 vacuum cleaners and three suction polishers was investigated. Bacteria-carrying particles were counted by means of a slit-sampler operating inside a large polythene reservoir in which stationary samples of exhaust air were collected from a polythene wind-tunnel in which the cleaners were operated. Serial samples of 5 cu. ft. were collected from machines before and after a 30-min. continuous run, during which the airflow was regularly interrupted every 15 sec. in order to stress the filters.

With one exception the vacuum cleaners gave colony counts not exceeding two in 10 cu. ft., and retained not less than 97% of the number of airborne bacteria found in the room air immediately before the test. One machine consistently gave counts of up to five colonies, retaining only 90% of the aspirated organisms. Suction polishers gave counts up to 10 colonies, with retention between 90% and 94%.

The aerial disturbance caused by vacuum-cleaner exhausts was also investigated; slit-sampler counts following the discharge of exhaust inside a small room showed that with open jet exhausts there was an immediate rise and fall in the number of airborne bacteria, followed by a slow bacterial fall-out over the next hour. When the speed of the airstream was reduced by means of a diffusing device at the outlet, no sign of disturbance was detected.

Silicosis

J. GOUGH (Cardiff) said that the latest theory of silicosis was the immunological one. Webster (1954) introduced it. A good summary of the present position is given by Vigliani and Pernis (1959). Pernis and Pecchiai (1954) showed that silicotic nodules contain 60% globulins including β and γ globulins. These occur as amorphous material in silicotic nodules. Electron microscopy shows collagen fibres embedded in the amorphous substance, the structure thus differing from simple scar tissue. Antweiler and Hirsch (1957) found that quartz coated with homologous serum albumin leads to the formation of antibodies when injected into laboratory animals. These antibodies can be detected by agglutination tests. Injected quartz acts as an adjuvant to the formation of antibodies in unrelated immunological reactions, e.g., the resistance to typhimurium infection is increased in mice prevously injected with tridymite (Pernis and Bolis, 1958). The relationship of an unrelated immunological reaction to silicosis was studied by Powell and Gough (1959), who showed that the immunization of rabbits with horse serum resulted in subsequently produced silicotic lesions being larger and more clearly demarcated and more collagenous. The anti-egg-albumen production in rabbits previously injected with tridymite was five to six times higher than in control rabbits (Ghiringhelli and Pernis, 1958). These observations are pertinent to the observation of Caplan (1953) that in rheumatoid miners the lung lesions are radiologically different from those of non-arthritic miners. Gough, Rivers, and Seal (1955) identified a corresponding difference in the pathology of the lesions, showing that pneumoconiosis renders the lung vulnerable to the nodular rheumatoid process. This may be an adjuvant immunological effect.

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Pneumoconiosis and Rheumatoid Disease

ANTHONY CAPLAN (Cardiff) said that when a workman exposed to a dust hazard suffers from rheumatoid disease, he may develop a specific type of lung lesion. The lesions are nodular, about 0.5–1 cm. in diameter, and distinguishable histologically from P.M.F. and classical silicosis. The lesions tend to conglomerate, and undergo liquefaction necrosis, fibrosis, and calcification.

The radiological appearances are characteristic and recognizable. Suddenly multiple, well-defined, round opacities, 0.5–5 cm. in diameter, appear, distributed throughout both lung fields. The background of simple pneumoconiosis is often slight or absent. The opacities usually increase in size and number, and crops of fresh lesions may appear at intervals of a few months. Cavitation is common in the active phase, and calcification may occur in older lesions.

Most frequently the development of opacities coincides approximately with the onset of arthritis; in a few cases the opacities precede the arthritis or the arthritis precedes the opacities by as much as six to 10 years. There is no relationship between the severity of arthritis and the extent and type of the radiological picture.

Epidemiological studies have shown that neither exposure to dust nor complicated pneumoconiosis is of any importance in the aetiology of rheumatoid arthritis.

The symptomatology is similar to that found in massive fibrosis, although there is often a surprising

absence of constitutional symptoms—even when extensive cavitation is present—and only slight impairment of lung function even in the presence of widespread opacities.

Antituberculous chemotherapy and/or corticosteroid therapy have failed to affect the progression of the lung lesions.

The aetiology of the lesions remains obscure. The reaction of the lung to the presence of even minimal deposits of dust or dust and tuberculosis is obviously influenced by a rheumatoid factor. This factor has known agglutination properties. It is, therefore, possible that rheumatoid pneumoconiotic lesions may be due to an abnormal immunological process.

Medico-legal Aspects of Pneumoconiosis

W. R. L. JAMES (Cardiff) referred to parts of the relevant acts and statutory instruments, including the Workmen's Compensation Acts, the Industrial Injuries Act, the Pneumoconiosis and Byssinosis Benefit Scheme, and the coroners' acts and rules.

Reference was made to the appropriate procedure for a doctor who suspected that his patient had died from pneumoconiosis.

The coroner's functions in pneumoconiosis cases were described, including his approach to the pathologist, his custody of tissues, and his possible procedures after receiving the pathologist's report.

The functions of the pathologist in pneumoconiosis cases were described, with his duties and privileges at the necropsy. Mention was made to the problem of the disposal of the body and how the pathologist could facilitate the necessary formalities.

Specific problems in assessing the cause of death were discussed, and the relationship of pneumoconiosis to some common natural diseases was indicated.

The constitution and functions of the pneumoconiosis medical panels were described, with emphasis on their duty to assess entitlement to death benefit.

Pathology of the Lungs in Beryllium and Nickel Workers

W. JONES WILLIAMS (Mount Vernon Hospital and Radium Institute, Northwood, Middlesex). Beryllium is increasingly used in industry, including atomic energy production. The inhalation of beryllium salts may lead to an acute or chronic disease, with an overall mortality of 27%. The cardinal features are pulmonary, but it is a generalized disease.

Seven acute cases were studied, and the changes were non-specific tracheo-bronchitis and pneumonitis. Most patients recover completely and only 2% progress to the chronic form.

Chronic beryllium disease usually occurs without an antecedent acute phase. In a study of 52 chronic cases the histological lesion was found a non-caseating granuloma indistinguishable from Boeck's sarcoid and with identical inclusion bodies. Chronic beryllium disease leads to diffuse interstitial pulmonary fibrosis and death may ensue from cor pulmonale. Beryllium is not yet shown to be carcinogenic in man but is so in experimental animals.

Nickel refiners using the Mond process have an increased incidence of lung cancer. There is no recognized pneumoconiosis but some degree of interstitial fibrosis was found in five cases examined. Two showed "honeycomb" lung and four had pulmonary carcinoma together with recent bronchopneumonia. There was a minimal amount of carbonaceous dust and no evidence of silicosis. The diffuse fibrosis could be post-inflammatory but may be due to inhalation of mixed industrial dust, including metallic nickel. Two cases were analysed and showed 90 and 120 times the amount of nickel found in controls. The gas nickel carbonyl, an intermediary compound in the Mond process, is carcinogenic to laboratory animals.

"Cadmium Pneumoconiosis"

J. P. SMITH (Manchester) described how cadmium had been used extensively in industry during the past 30 years. Inhalation hazards arise from exposure to cadmium oxide dust or to cadmium fume liberated when the metal is heated and may be acute or chronic in onset. The fumes are odourless and have no immediate irritant effect and can be inhaled in fatal concentration without anyone being aware of the danger.

Acute poisoning develops a few hours after exposure to high concentrations of the fume and leads to dyspnoea and pulmonary oedema. Striking reactive changes are seen in the alveolar epithelium of fatal cases.

Chronic poisoning is insidious and follows repeated exposure over several years to moderate concentrations of fume or oxide dust. Emphysema develops which, in severe cases, progresses to respiratory failure despite removal from exposure.

Pulmonary Fibrosis after Exposure to Aluminium Powder

G. B. MANNING (Bolton) reported that in 1954 a man aged 22 years died from pulmonary fibrosis. He had begun to work in an atmosphere heavily contaminated with aluminium powder three years and five months before his death. The man who took over the identical job vacated by the previous case also died from pulmonary fibrosis in 1959, four years and nine months after starting this work. A third case of fibrosis of lung from the same works was first suspected from the radiological appearances of the chest before his place of employment was known. This patient had, in fact, only spent one year in that same factory (Mitchell, 1959).

Further investigations have shown that to date out of 20 men who were exposed to heavy concentrations of aluminium powder in the atmosphere for more than six months, two have died from pulmonary fibrosis, four have evidence of pulmonary fibrosis, 11 have normal chests, and three have refused examination. These investigations are being prepared for publication.

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Farmer's Lung

ROGER M. E. SEAL (Sully) said that the account of the disease was based mainly on cases from the west Wales area, when 41 new patients were seen in 1959 after the wet summer of 1958.

An acute form following a single heavy exposure, as after threshing, with dyspnoea and dry cough with a fine "spotting" radiologically, was described. This type apparently resolved in three to six weeks without treatment.

A progressive type, which followed repeated exposure, was described as characterized by severe exertional dyspnoea, a dry cough, and radiologically a diffuse opacity of the bases with a background of miliary mottling. Laboratory investigation revealed a hypergamma globulinaemia and diffusional impairment of oxygen exchange across the alveolar capillary membrane with a reduced pO_2 and pCO_2.

The histology of the acute and progressive types consisted of thickening of alveolar septa with small round-cell infiltration and with superadded epithelioid foci mimicking sarcoidosis.

Examples of a chronic end-result of farmer's lung were described which clinically and radiologically presented as diffuse fibrosis and honeycomb and cystic lung. The histology also was that of severe pulmonary destruction with fibrosis and collagen-lined cystic areas.

The aetiology was discussed and the condition was differentiated from sarcoidosis and pulmonary moniliasis and aspergillosis. The mycology and bacteriology of good and mouldy hay were contrasted and it was considered that the disease represented a hypersensitivity reaction diffusely in the lung to one or more antigens in mouldy hay.

Further Studies of Anaemia in Pregnancy

C. GILES (Stoke-on-Trent) reported that in a controlled trial of prophylactic folic acid during the last trimester of pregnancy, 758 women who received oral folic acid and iron showed significantly higher haemoglobin levels at term than 721 women treated with iron alone. Megaloblastic anaemia developed in 20 cases of the control group but in none of the patients treated with folic acid. This trial supports the concept that folic acid depletion tends to occur in late pregnancy, even in the absence of megaloblasts in the marrow.

Analysis of 156 cases of megaloblastic pregnancy anaemia, diagnosed over a period of three years, suggests that an inadequate diet and intestinal malabsorption are important factors predisposing to megaloblastic anaemia. Abnormally high faecal fat excretion was found in 12 out of 41 cases which were investigated, but in none of 17 normal pregnant women.

When megaloblastic anaemias of pregnancy were classified according to the types and relative numbers of megaloblasts in the marrow, an abnormal blood group distribution, with a significant increase of blood group A, was found in cases with the more advanced degrees of megaloblastosis. These findings suggest that folic acid deficiency tends to occur in a large number of women in late pregnancy, but only in a certain group, with an inherited predisposition, will folic acid depletion result in florid megaloblastic anaemia.

Direct and Indirect Precipitin Tests in Thyroid Disease

J. M. MOORE (Stobhill Hospital, Glasgow) described a serum precipitin test. This, giving a positive result with thyroglobulin, is generally considered to indicate major auto-immune thyroiditis. However, in a substantial proportion of patients with this disease the precipitin test is negative.

Two methods, neither of them original, were used to render this type of test more sensitive: (1) Four-fold concentration of serum: three sera, out of 20 tested, gave a definite positive with concentrate when the result with neat serum was negative or doubtful. (2) The use of adjuvant sera. Sera from cases of auto-immune thyroiditis giving a band of clearing on Ouchterlony plates instead of a band of precipitate have already been described. Two such sera were used to test precipitin-negative sera for the presence of a component which would convert the clear band to a precipitin band, either by diffusion of test and adjuvant sera towards one another on Ouchterlony plates or by mixtures.

Twenty-nine per cent. (15) of sera—precipitin-negative by routine, or direct, test—from a series of cases of Hashimoto's disease and primary myxoedema gave positive results with the indirect test. The indirect test was positive in some cases with a weak direct precipitin but not in any with a strong direct precipitin. Eighty-eight direct-negative sera from patients without thyroid disease and 33 from patients with thyroid disease not of immune type gave negative indirect results. Four negative direct and positive indirect results were obtained in women with thyroid C.F. antibody in their serum but without clinically-apparent thyroid disease.

First Experiences with the Autoanalyser

M. LUBRAN (West Middlesex Hospital). Micromethods for urea and glucose employing, respectively, a 1 in 10 and 1 in 20 dilution of blood may be run satisfactorily at 40 an hour, if a water wash is incorporated between samples. This gives an effective running rate of 20 an hour and prevents a low-value sample being contaminated by a preceding high one. The alkaline phosphatase procedure is very satisfactory, but the acid phosphatase method recommended for the apparatus measures only total acid phosphatase. Formaldehyde-stable acid phosphatase can be measured by including formaldehyde in the buffer substrate. However, this sometimes gives rise to precipitation of protein and the recorder must be carefully watched to notice when this happens.

During the average working day, the "autoanalyser" saves the time of at least one trained technician and gives, consistently, a higher standard of accuracy than

the technicians. As no glassware is used, the running costs are less than the labour costs involved in manual methods. Most emergency work can be fitted in to the machine's programme, but a small number of manual determinations will have to be carried out for true emergencies and for night work.

The "autoanalyser" is suitable not only for large laboratories but for smaller laboratories, where five or six different determinations, involving about eight samples each, may be carried out by one technician in a working day.

The Application and Implications of Bone Marrow Grafting in Man

J. G. HUMBLE (Westminster Hospital Medical School, London University). Modern therapy, especially modern chemotherapy for malignant disease, tends to destroy the bone marrow and lymphatic system. As bone marrow suspensions will re-seed the aplastic marrow produced by irradiation in laboratory animals, the application of this method has been studied in man. Methods of obtaining bone marrow by puncture, of storing it at −79° C., and of administering it safely to the recipient have been worked out. In six of eight patients treated by whole chest irradiation marrow function was restored to the sternum by stored autologous marrow. In four out of nine cases treated by massive chemotherapy marrow function was similarly restored. The other patients died before marrow function could be restored. Some evidence of the effect of the treatment on the tumours was noted. The danger of infection, especially of moniliasis, was emphasized. Despite recent criticism, the results suggest that a true re-seeding of the marrow spaces takes place. Using homologous transplants, three cases of aplastic anaemia and five of acute leukaemia have been treated. No remissions have been obtained although the last leukaemic child treated was showing haematological recovery when overwhelming moniliasis supervened. It was emphasized that the use of stored autologous marrow permitted a much greater margin of safety in the vigorous treatment of malignant disease processes.

Transplantation of Foetal Haemopoietic Cells

H. E. M. KAY (Royal Marsden Hospital, London). The advantages and disadvantages of using foetal haemopoietic cells in marrow replacement therapy were discussed with particular reference to the problem of tolerance by the graft towards the host tissues.

Treatment of cases of leukaemia, of chemically-induced and idiopathic bone-marrow aplasia, and of agammaglobulinaemia was described and the reasons for success or failure analysed.

Finally the problems of foetal cell embolism and the use of foetus-to-foetus transplants were considered.

On the Rationale of Bone Marrow Grafting

L. G. LAJTHA (Radiobiology Laboratory, Churchill Hospital, Oxford) said that, considering the population kinetics of the bone marrow in respect of regeneration, and the available *x*-ray dose response curves of mammalian cells (in respect of reproductive integrity) it appears that isologous marrow grafting only saves an equivalent time for one complete mitotic wave in the regeneration of the integer stem cells. Available experimental as well as theoretical evidence suggests that a similar time-saving might be achieved by simpler methods, i.e., venesection and/or T.A.B. injections.

The efficiency of shielded marrow in cases of partial body irradiation was stressed. It is suggested that, in man, marrow grafting is a procedure which could be avoided by simple substitution therapy and stem cell stimulation.

Intestinal Biopsy in Coeliac Disease

A. H. CAMERON (Birmingham) said that the histological findings in duodenal or jejunal biopsies from 37 children were described. The ages ranged from 3 months to 14 years. Using a technique for estimating the relative surface area of the mucosa, the biopsies were separated into two groups. The first showed no significant loss of mucosal surface area. They were obtained from 11 patients, one of whom was a case of coeliac disease who had responded well to a gluten-free diet. The other 10 showed no clinical evidence of coeliac disease. Of the remaining 26, the clinical diagnosis was coeliac disease in 21, probable coeliac disease in four, and uncertain in one. Only one was on a gluten-free diet at the time of biopsy. This group was subdivided according to the severity of the histological appearances. The histological grading may be of value in evaluating response to therapy. It was concluded that biopsy is a safe and important method of investigation in steatorrhoea of childhood.

Coagulation Tests on Capillary Blood

KATHARINE M. DORMANDY and R. M. HARDISTY (The Hospital for Sick Children, Great Ormond Street, London) presented a scheme for the routine investigation of coagulation disorders using free-flowing capillary blood. Blood, 0.2 ml., is taken into a buffered citrate-saline mixture and used for the "prothrombin and proconvertin" test of Owren and Aas (1951) and and thromboplastin screening test of Hicks and Pitney (1957); the clotting time of the capillary blood (Dale and Laidlaw, 1911–12) is also determined. These three tests are used as screening procedures; accurate identification of any defects revealed can be achieved by assay procedures carried out on the same specimen of blood.

Details of the methods were given, and examples of results obtained in various coagulation disorders shown. The sensitivity and accuracy of the tests were indicated by comparison with the results of tests performed on venous blood obtained simultaneously.

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BOOK REVIEWS

A Biochemical Approach to Pathology. By M. J. R. Dawkins and K. R. Rees. (Pp. viii+128; 30 figures. 18s.) London: Edward Arnold. 1959.

Biochemistry (using the word properly as denoting an academic discipline) is a subject, a knowledge of which is indispensable to an intelligent understanding of medicine to-day. It is increasingly needed for competent diagnosis, for rational therapy, and for worth-while original work. The unfortunate habit of referring to chemical pathology as "biochemistry" has led some to think of biochemistry as a collection of receipts—suitable for a cook book—and this confused thinking has led at times to neglect of the important medical aspects of the subject. To this must be added a divorce between medicine and biochemistry and the view that biochemistry is fundamentally a "science" subject. It must be a matter for regret that the teaching of biochemistry is so often in the hands of teachers who, however eminent, lack medical training. As a result the importance of biochemistry to the medical practitioner is often not brought home to the student: he appreciates that the subject is one of importance to medical research but fails to see how it can help in the day-to-day practice of medicine or how it can help him to understand disease. The authors of this book say, "This book was written because we feel that advances in pathology will depend more and more on a detailed knowledge of cell metabolism." The book will certainly help to awaken interest in cell metabolism, but it will do more than that: it will help to awaken interest in the whole field of disordered biochemistry in relation to disease.

The book sets out in simple language some of the results of applying biochemistry to disease processes. Very little knowledge of chemistry is demanded and the book is very readable. It can be recommended confidently to students; there must be many registrars and consultants in pathology or general medicine who would benefit from reading it. It can be read in an evening.

ARTHUR JORDAN.

Biochemistry of Blood in Health and Disease. By I. Newton Kugelmass. (Pp. ix+543; 20 figures. £6 6s.). Oxford: Blackwell. 1959.

This book is divided into three parts: Blood as the transport system, as the regulatory system, and as the defence system. The first two are usually regarded as belonging to biochemistry, the last to haematology and serology.

In the reviewer's opinion, the attempt to relate "transport" and "regulation" to changes in the blood, with only very scant reference to such matters as renal function, gives an unbalanced picture, and many parts are very difficult to understand.

There is an enormous amount of information in the book, but the general impression given is that it is a rather uncritical re-write of the author's jottings and lecture notes; for instance, protein changes in multiple myeloma are referred to under several headings in the sections on "plasma protein excesses" and "plasma protein defects" on pp. 50–55, but, even on reading the whole sections carefully, the reader is left guessing as to what changes are likely to be met with in this condition or their significance.

The suggestion (p. 178) that "Cushing's syndrome with alkalosis produced in prolonged A.C.T.H. or cortisone therapy may be corrected by adrenalectomy or removal of a possible cortical tumour" is typical of the frequent careless writing. The amount of A.C.T.H. in normal blood is recorded as "0.5 mμ/100 ml." in Table XII, 2, on p. 267, as "15 μg./100 ml." in Table XII, 1, and in the table of normals on p. 502; mμ presumably means milli-units, though it is defined in the tables of abbreviations as millimicrons, and the relation of mμ to μg. is nowhere explained.

Throughout the book there are innumerable other examples of carelessness, and these, apart from the very high price, £6 6s., will tend to limit its general usefulness.

E. N. ALLOTT.

Laboratory Manual of Pediatric Micro-Biochemical Techniques. By Donough O'Brien and Frank Ibbott. (Pp. 164.) University of Colorado. 1959.

The methods in this paper-back manual from the Department of Pediatrics, University of Colorado, are not, in spite of the title, confined to paediatric applications, and could be applied to any hospital population. A few unusual items, e.g., the test for chondroitin sulphate B in the urine of patients with gargoylism, are included. The text is strictly confined to details of technique. Normal ranges are stated, but no information is given about the interpretation of results, and often the principles of the methods are omitted. Most of the blood analyses require 0.1–0.2 ml. volumes, no ultramicro procedures being given.

In a book of this type more might have been said about possible pitfalls in technique. Nevertheless this is a useful collection of methods which can be recommended for their simplicity and can be employed on British equipment without modification.

W. H. R. AULD.

Heavy Metals and the Brain. By John N. Cumings. (Pp. vii+161; 4 figures. 32s. 6d.) Oxford: Blackwell Scientific Publications. 1959.

This book consists of a thorough review of the literature (there are some 900 references) dealing with copper, mercury, and lead and their relation to the

brain. The author has thus excluded the peripheral nervous system from the discussion and only barely mentions the spinal cord.

For each of the three metals there is an historical note, a discussion of the clinical features of the various metabolic abnormalities or intoxications, and sections on clinical pathology, histopathology, and treatment. Copper metabolism receives the most detailed consideration and this chapter includes a valuable discussion of normal as well as abnormal copper metabolism. There is an almost complete list of published cases of hepato-lenticular degeneration. The chapter on mercury contains a section on pink disease.

This is a most useful book for anyone wishing to become acquainted with the literature concerning these three metals and the brain.

SABINA STRICH.

The Effect of Advancing Age Upon the Human Spinal Cord. By the late L. Raymond Morrison in collaboration with Stanley Cobb and Walter Bauer. (Pp. x+127; 86 figures. 48s.) Harvard University Press (London: Oxford University Press). 1959.

This posthumous publication, the completion of which we owe to Dr. Stanley Cobb and to Dr. Walter Bauer, will be useful to those who are uncertain as to the significance of certain histological changes in the spinal cord. The illustrations, which are culled from a series of 31 specimens ranging from the second to the ninth decades of life, show the range of changes which may be regarded as normal: death in most instances was due to medical causes, which one assumes were regarded as being of a type not likely to have given rise to changes in the cord.

The second half of the book is in fact an atlas of the spinal cord taken at each segmental level, special prominence being given to the cell populations. These are all made on a 24-year-old subject.

W. H. MCMENEMEY.

The Effect of Pharmacologic Agents on the Nervous System. [Res. Publ. Ass. nerv. ment. Dis., Vol. xxxvii.] (Pp. xi+488; illustrated. 108s.) London: Baillière, Tindall & Cox. 1959.

This volume, the 37th of the series, is as valuable as those that have preceded it. There are recorded in this book 26 separate articles, together with the discussion each involved, which were given in 1957 as the Proceedings of the Association for Research in Nervous and Mental Disease. Most of the chapters are directly related to the effects and actions of various pharmacological agents in neurological and psychiatric conditions, both as they occur in man or can be induced in animals. While most of the chapters are extremely good, special note must be made of the communications by Dr. Melvin Yahr on anticonvulsants, Dr. S. A. Jervis on metabolic aspects, and Dr. H. E. Himwich on stimulants. Anyone interested in this field of neurology and psychiatry must read this authoritative book and many workers in other fields would profit by a consideration of some of the methods that have been employed in the various studies related therein.

J. N. CUMINGS.

A Symposium on the Evaluation of Drug Toxicity. Edited by A. L. Walpole and A. Spinks. (Pp. xi+138; 9 plates. 25s.) London: J. & A. Churchill. 1958.

During the last few years physicians and the manufacturers of drugs have shown anxiety at the increasing frequency of serious illness and death directly attributable to the administration of drugs. This is shown by the appearance of three symposia on this subject within three years and by many discussions in medical societies. The first symposium, "Nebenwirkungen von Arzneimitteln auf Blut und Knochenmark" (Stuttgart, 1957), organized by Hoffman-La Roche, was attended by 54 specialists, who spoke in German, and 19 papers were read and discussed. The second, organized by the C.I.O.M.S., "Sensitivity Reactions to Drugs" (Oxford, 1958), was a smaller meeting with only 19 participants, who spoke in English. The most recent, now under review, was organized by I.C.I. Ltd., with only nine principal speakers but 46 participants, of whom only two were not British. These three volumes give a general picture of the Swiss-Austrian-German-Scandinavian approach, British-French-American-Swiss, and British approaches to the problem.

The I.C.I. volume is at once the smallest and the most exacting of the three, since the principal contributors are experimental pharmacologists and physiologists, and the first session, inaugurated by Professor E. J. Wayne, was continued by members of the staff of I.C.I. (Pharmaceuticals) Ltd. and the American Cyanamid Co., who explained their methods of assessing the efficiency, safety, and toxicity of new drugs. This is a most valuable contribution to the literature, for it is a critical exposition of the problem and of the way it is now being studied. Perhaps the most important paragraph is that of Dr. G. E. Paget:

> "I think every biologist and clinician should have it clear in their own minds, and make it clear to their chemical and commercial colleagues, that every time a new drug is tried in man we are conducting an experiment the outcome of which, both therapeutically and toxicologically, must be in considerable doubt no matter how much experimental work has been done."

The second session deals with the effect of drugs on specific organs, structures, or responses—allergic response, bone marrow toxicity, liver toxicity, and action on subcellular structure—and utilizes information from humans as well as animals. It is impossible to summarize the wealth of useful information in these pages, and it is presented in ways which continually stimulate the imagination and constantly reveal new possibilities. This is not a book for the laboratory but for the study; and those who read it will learn some useful clinical medicine as well as academic and clinical pathology.

GEORGE DISCOMBE.

Weight Gains, Serum Protein Levels, and Health of Breast Fed and Artificially Fed Infants. [*Spec. Rep. Ser. med. Res. Coun. (Lond.) No. 296.*] By B. Levin, Helen M. M. Mackay, Catherine A. Neill, V. G. Oberholzer, and T. P. Whitehead. (Pp. 154; 69 figures. 16s.) London: H.M.S.O. 1959.

This report is based on 946 infants and children studied at the Salvation Army Mothers' Hospital and Queen Elizabeth Hospital, London. The aim was originally that of determining the optimum protein content of infant diets, and this involved assessing the normal content and variation of serum protein levels in full-term and immature infants.

The authors have attempted to overcome the great variation found in what are termed "premature" babies, and they were forced to classify their babies on the basis of birth weight.

The report contains a great deal of information on the weight gain of infants on different diets, which is probably of primary interest to the paediatrician and infant welfare officer. The biochemical studies of the serum proteins suggested that lower birth weight was associated with lowered serum proteins. Low serum protein levels seen in the premature infants (based on weight) is overcome by the end of 12 months after birth.

Gamma globulin levels show great variation, particularly in the early months of life, adult levels being reached around puberty. Factors such as type of feeding, season of the year, or mild infection did not appear to affect protein levels.

The authors provide a table giving the total protein, albumin, and globulin blood levels from birth to up to 18 months after birth. These figures are probably likely to be the part of the report of most value to the clinical pathologist.

This investigation has involved a great deal of work, of which the delay of approximately 10 years between the survey and date of publication is perhaps an indication.

JOHN L. EMERY.

Clinical Toxicology. By C. J. Polson and R. N. Tattersall. (Pp. xi+589. 42s.) London: English Universities Press. 1960.

Any book associated with the name of Polson will always include an adequate bibliography and this is no exception. On the other hand, it does not include any analytical techniques, but to include those as well as the clinical aspects would be impossible in a book of this size. It contains extremely comprehensive information about the poisons most likely to be seen in practice and should be invaluable to anyone in hospital who wishes to confirm his suspicions of a suspected case and to check on the appropriate treatment. There is, however, one great difficulty when writing books on clinical toxicology, and that is that the symptoms of many kinds of poisoning resemble those of natural illness at one stage or another. This fact, coupled with the fact that many doctors are unsuspicious, makes it not surprising that it is common for cases of poisoning to be missed. This means that in books on clinical toxicology, if they are to be comprehensive, such symptoms as nausea, diarrhoea, abdominal pain, and so on, which are common features in most cases of poisoning, must be repeated, and consequently the important aspect of the diagnosis of any case of poisoning, the appreciation of the unusual, is not sufficiently stressed. For example, in cases of alcoholic poisoning if certain eye signs have been noticed, there is a strong possibility that the patient has taken methyl alcohol. This is the art of toxicology and requires medical knowledge. The analytical techniques are another matter, because they require considerable technique, skill, and experience outside the scope of most medical practitioners. In the United States there are many experts who describe themselves as "toxicologists" but who are, in fact, analytical chemists, whereas there are forensic pathologists who know a great deal about the post-mortem appearances and clinical aspects of poisoning, but do not call themselves toxicologists. A happy compromise will have to be reached in the future by which those with medical qualifications will have to interpret the results of the non-medically qualified analyst, each admitting that he cannot assume the mantle of the other.

The section on the law of poisons is quite adequate for the medical profession but perhaps a little superficial for lawyers or pharmacists. Nevertheless, this book can be recommended to pathologists who have to assist coroners and to clinicians who are likely to be faced with cases of poisoning in hospitals.

F. E. CAMPS.

Eight Colloquia on Clinical Pathology. 3rd International Congress of Clinical Pathology, Brussels, July, 1957. Edited by Maurice Welsch, Pierre Dustin, and Jacques Dagnelie. (Pp. iv+829; illustrated.) Brussels: Presses Académiques Européennes. 1958.

The report of the Third International Congress of Clinical Pathology, held in Brussels in July, 1957, has finally appeared, and although the editorial date appears to be 1958, this large volume of 829 pages was distributed to the participants less than three months before the Fourth International Congress is due to start in Madrid. Although one realizes the enormous amount of labour that the editors put into such a volume, this seems an unnecessarily long interval, particularly as many of the papers and discussions presented at this congress had already been published. While this volume is, therefore, an excellent record of previous work, it can hardly be used as an up-to-date reference book on any of the subjects concerned. This seems a pity, and it would be an immense help to pathologists—both those who attend the congresses and those who would like to read the papers—to have publication expedited in future.

A. G. SIGNY.

J. clin. Path. (1960), **13**, 279.

RETICULO-ENDOTHELIAL SARCOMA ARISING IN THE NOSE AND PALATE (GRANULOMA GANGRENESCENS)

BY

E. W. WALTON

From the Department of Pathology, Queen's College, Dundee, University of St. Andrews

(RECEIVED FOR PUBLICATION JANUARY 12, 1960)

Granuloma of the nose which does not heal is a tripartite disease, including classical malignant granuloma, Wegener's granulomatosis, and a third group with neoplastic characteristics (granuloma gangrenescens). Five cases of this third type are described and 20 others from the literature are tabulated. The primary lesion is in the nasal or palatal region; after a latent period, metastases develop in many organs. The histological features are those of malignancy. Reasons are given for regarding the condition as a sarcoma of the reticulo endothelial system.

A recent survey of 23 cases of nasal granuloma which did not heal (Walton, 1959) has shown that this disease is a tripartite one including cases of non-specific granulomatous inflammation (malignant granuloma), others associated with polyarteritis nodosa (Wegener's granulomatosis), and others more closely resembling a neoplasm (granuloma gangrenescens). In this third and least common group, a prolonged nasal or palatal ulceration is usually followed by the development of metastatic lesions throughout the body.

Five cases in which sarcomatous tissue was found are described.

Case Reports

Case 1.—A labourer, aged 49, was admitted to hospital in November, 1954, with pain and ulceration in the mouth for five weeks. The whole of the right side of his face, nose, and upper lip was swollen. An ulcer on the hard palate, 3 cm. in diameter, had necrotic bone in the base. The nasal mucosa was red and thickened. He was febrile but otherwise well. Bacteriological and serological tests were negative. Biopsy of the palatal ulcer showed necrotic granulation tissue but no evidence of malignancy. He was treated with antibiotics, iodine, and arsenicals, but the ulcer enlarged until the hard palate and floor of the nose were destroyed. The diagnosis of malignant granuloma was made and a course of *x*-ray therapy was begun two months after admission. The improvement was dramatic and three months later the lesion had healed.

The patient remained well for eight months, when the right testis enlarged and became painful and the vision in the right eye began to fail. On examination the right retina was detached, and ulceration in the upper nasal cavity and a mass in the ethmoid sinuses were seen. The palatal lesion was still healed. The right testis was firm and slightly enlarged. Orchidectomy was performed and the testis found to be infiltrated with greyish tissue. Despite *x*-ray therapy to the sinuses and right orbit, the loss of vision increased and became bilateral. He developed small ulcers in the skin of the arms and trunk, lapsed into coma, and died, 16 months from the onset of the illness.

Summary of Necropsy.—The body was emaciated and numerous small red spots, some superficially ulcerated, were present on the skin of the arms and trunk. The hard palate and alveolar process of the maxillae were almost completely destroyed. The nasal mucosa was diffusely thickened and ulcerated. The right eye showed almost complete detachment of the retina and haemorrhage behind it; the orbits were not examined. The thyroid gland was enlarged, irregular on section, and showed a white nodule in the right upper pole. There was a bilateral bronchopneumonia and extensive fibrosis of the myocardium. Numerous acute ulcers, shallow but with heaped-up edges, were present in the stomach and proximal ileum. The capsular and cut surfaces of the liver showed numerous tumour nodules measuring up to 1 cm. The pancreas was diffusely fibrotic but contained no tumour. The right kidney was enlarged (210 g.) and diffusely infiltrated by neoplastic tissue, mottled and yellow-brown. Similar tissue was present in the left epididymis. There was no lymphadenopathy. All other organs and tissues appeared normal.

Case 2.—A storekeeper, aged 56, developed swelling of the right side of the nose in the summer of 1956. Despite antibiotic treatment it persisted and by October there was an extensive nasal ulcer with erosion of the septum, the right lateral nasal wall, and the turbinate bones. Repeated biopsies showed granulation tissue but no evidence of neoplasia. Bacteriological and serological tests were negative.

By February, 1957, the right face and jaw were swollen and there was a 2½ cm. ulcer above the right nasal fold which penetrated deeply into the nasal cavity. A radiograph of the skull showed partial destruction of the nasal septum. After a course of *x*-ray therapy the ulcer began to heal by granulation and by June it had decreased in size. The next month, however, the left side of the nose swelled and the facial ulcer began to enlarge. On his admission in August, the external ulcer measured 4½ cm. in diameter; the palate was eroded, and he had gross foetor oris. A radiograph of the skull showed destruction of the medial walls of both maxillary antra. He slowly became confused and dyspnoeic, developed tremor of the facial muscles and tongue, and died on August 19, 14 months after the onset of the illness.

Summary of Necropsy.—The body was emaciated. A large perforating ulcer had destroyed much of the right nostril and the nasal septum, turbinate bones, palate, and both maxillary antra were eroded. The frontal, ethmoidal, and sphenoidal sinuses and the base of the skull appeared healthy. The brain (1,350 g.) appeared externally normal but on section showed three areas of haemorrhage, each about 1 cm. in diameter, in the pons, left cerebellar lobe, and the left post-central gyrus. The lungs showed a bilateral lower lobe bronchopneumonia. There was moderately severe atheroma of the aorta, its main branches and the coronary arteries. All other organs and tissues appeared normal.

Case 3.—A mentally defective spinster, aged 30, was admitted to hospital in July, 1956, having had a "carbuncle" of the left nostril for five weeks. On examination the only abnormality found was a granulating ulcer involving the left nostril, the nasal septum, and the upper lip; biopsy of this showed granulomatous inflammation with no evidence of neoplasia. Treatment with antibiotics had little effect on the course of the disease. Five months later the lesion had progressed and now affected the upper lip, nose, and both cheeks (Fig. 1). The eyes were swollen, the left being almost closed. Shortly afterwards she became comatose and died, six months after the onset of the illness.

Summary of Necropsy.—The body was emaciated. A large fungating tumour replaced the nose and upper lip. There was ulceration in both nostrils, with some erosion of the bony skeleton. The upper gum and palate were swollen and indurated. The lower air passages were normal. The lungs (right 700 g., left 680 g.) were oedematous and patchily consolidated. The liver and spleen were congested and there was a haemorrhagic cystitis. The brain was not examined. All other organs and tissues were normal, there being nothing to suggest metastases.

Case 4.*—A housewife, aged 37, was admitted to hospital in March, 1958; for 10 months she had had

*This case is to be published in detail elsewhere (Dawes and Wort, 1960).

FIG. 1.—Case 3, five months after the onset. A fungating mass has destroyed the nose and the remainder of the face is greatly swollen.

right-sided nasal obstruction and one week previously had developed swelling of the right side of the face and gum. On examination the right nostril was found to be blocked by a granulomatous mass and numerous small nodules were noted in the skin of the forearms and thighs. A nasal biopsy showed infiltration by sarcomatous tissue. After deep *x*-ray and steroid therapy the swelling of the face and nasal granuloma both diminished. In June, however, she developed a sore throat, a painful cough, cervical and axillary lymphadenopathy, and more skin nodules. In August, peritonitis supervened and at laparotomy a perforation in the distal ileum was closed. A week later she died in peripheral circulatory failure.

Summary of Necropsy.—The body was well nourished: numerous firm nodules were present in the skin of the arms, chest, and thighs. A granulomatous ulcer covered the right lateral wall of the nasal cavity and extended medially on to the nasal septum. The lungs were both oedematous (2,300 g.) and each contained several round nodules, firm greyish-yellow, sharply defined, and about 2 cm. in diameter. There was a healing peritonitis and the small bowel, throughout its length, contained multiple firm nodules in the submucosa, each pale grey and measuring up to 2.5 cm. in diameter. The liver was pale but otherwise normal. The other organs and tissues examined, including the brain, showed no significant abnormality.

Case 5.—A labourer, aged 67, developed pain and swelling of the left cheek with blockage of the nose, in the spring of 1958. He was otherwise well. On examination one month later there was extensive ulceration of the lateral wall of the nose, over the turbinate bones and the septum. As antibiotics did not improve his condition, a biopsy was taken. This was diagnosed as being infiltrated by a sarcoma of the reticulo-endothelial system. A course of radiotherapy resulted in apparent healing of the lesion: when last seen nine months later he was well.

Summary of Clinical Features and Macroscopic Appearances at Necropsy

The disease in each case followed a constant pattern. The initial lesion, an ulcer of the nose or palate, persisted despite chemotherapy or antibiotic treatment, but *x*-ray therapy in each case produced a retrogression or even apparent cure. The original biopsies were nearly always non-specific. Bacteriological and serological tests were negative and the blood picture was always normal. Cases 1, 2, and 3 were originally regarded as examples of the so-called malignant granuloma, but further study of the reported cases of this non-specific granulomatous inflammation made it obvious that the present cases belonged to the truly neoplastic group (in the past called granuloma gangrenescens), and this enlightenment allowed a provisional clinical diagnosis in Cases 4 and 5, confirmed in both by nasal biopsy.

The local lesion in each case produced erosion of the cartilaginous and bony framework of the nose and palate, and, in the cases that came to necropsy, neoplastic tissue was found here. Spread to the skin of the face occurred only in Cases 2 and 3, in the latter a large fungating tumour mass developing. In Cases 1, 2, and 4, metastases were found in many organs and tissues, usually the lymph nodes, skin, liver, kidney, spleen, and lung. More unusual sites for metastatic growth were also involved, namely, heart, gonad, thyroid. The tumour deposits were usually diffuse infiltrates of homogeneous pale tissues, less frequently discrete nodules. Sometimes areas of necrosis or haemorrhage were present as in the cerebral lesions in Case 2.

Histological Features

These are based on the biopsy material from the initial nasal or palatal lesions in each case, and necropsy tissue from Cases 1 to 4. Unfortunately, the nasal material was usually small and fragmented, consisting largely of ulcerated granulation tissue, with areas of necrosis. Nevertheless, in the depths of the tissue, neoplastic infiltrations were identified in each case. In brief, these consist of sheets of quite uniform cells with round or oval hyperchromatic nuclei and little cytoplasm. Mitotic figures are fairly frequent, up to 1 or 2 per high-power field.

TABLE I

CLINICAL AND PATHOLOGICAL FEATURES OF 24 NECROPSIED CASES OF RETICULO-ENDOTHELIAL SARCOMA ARISING IN THE NOSE AND PALATE

Case No.	Author	Sex	Age	Primary Lesion	Duration (Months)	Metastases
1	Kraus (1929), Case 2	M	30	Nose	23	—
2	Bonne and Lodder (1929), Case 1	M	42	Palate, nose	—	Liver, spleen, lung, heart, adrenal, stomach, ileum, pituitary, pia mater
3	,, ,, ,, 2	F	46	Nose	6	Lymph nodes, liver, pancreas, stomach, ileum
4	Derischanoff (1931)	F	62	,,	—	—
5	Berendes (1934)	F	31	,,	3	—
6	Hall (1934)	F	32	Palate, nose	4	Skin, lymph nodes, kidney, lung, voluntary muscle
7	Kanas (1936)	M	47	Palate	3½	Lymph nodes, pharynx
8	Tischnenko, Kroitschik, and Kusnetz (1936)	M	37	Nose	12	—
9	Bergqvist and Koch (1949), Case 2	M	27	Nose, palate	4	Skin, lymph nodes, lung, voluntary muscle
10	Rasmussen (1948)	M	29	Palate, nose	—	Skin, voluntary muscle, ? liver, kidney, spleen, lung
11	Thielemann and Pieczewski (1950)	M	47	Palate	3	Lymph nodes, heart
12	Vogel (1952)	M	30	Nose, palate	9	Skin, lymph nodes, liver
13	Corrêa and Elisabetsy (1954), Case 1	M	17	Palate	7	—
14	,, ,, ,, ,, 2	F	27	Nose	—	—
15	Calas and Bonneau (1958)	M	38	Palate	24	Salivary gland, ? intestine
16	Richmond, Weir, and Philip (1956)	F	44	Nose	11	Lung, ovary, ? brain, colon
17	Spear and Walker (1956)	M	43	,,	15	Skin, lymph nodes, larynx, trachea
18	Hultberg, Koch, Moberger, and Mårtensson (1957), Case 2	F	42	,,	13	Lymph nodes, liver, kidney, spleen, adrenal, uterus, pituitary
19	Lopes de Faria *et al.* (1957), Case 1	F	36	,,	20	Lymph nodes, liver, kidney, heart, uterus
20	Baker *et al.* (1958)	M	29	Nose, nasopharynx	10	Lymph nodes, liver, spleen, lung
21	Walton, Case 1	M	49	Palate	15	Skin, lymph nodes, liver, stomach, kidney, spleen, testis, thyroid
22	,, ,, 2	M	56	Nose	14	Lymph nodes, lung, brain
23	,, ,, 3	F	30	,,	6	—
24	,, ,, 4	F	40	,,	6	Skin, lymph nodes, liver, kidney, spleen, lung, heart, ileum, ovary, adrenal, thyroid

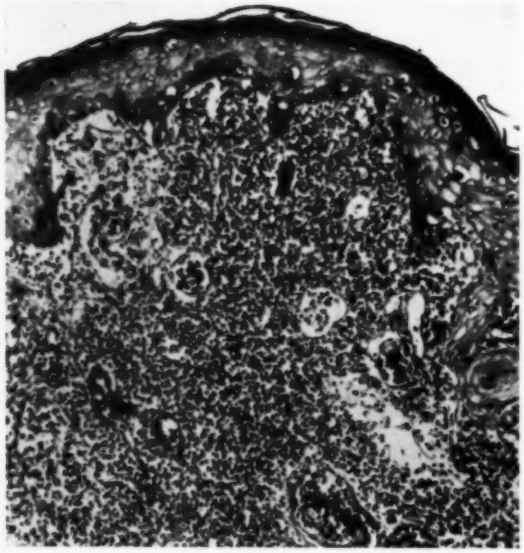

FIG. 2

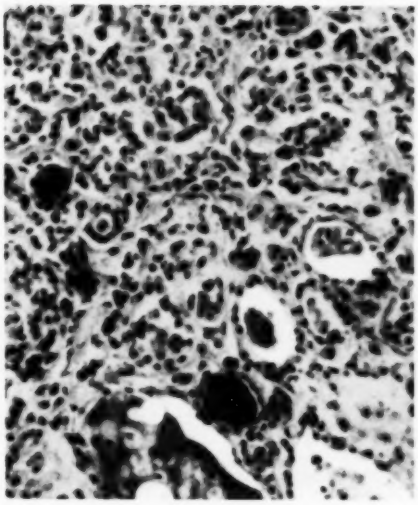

FIG. 3

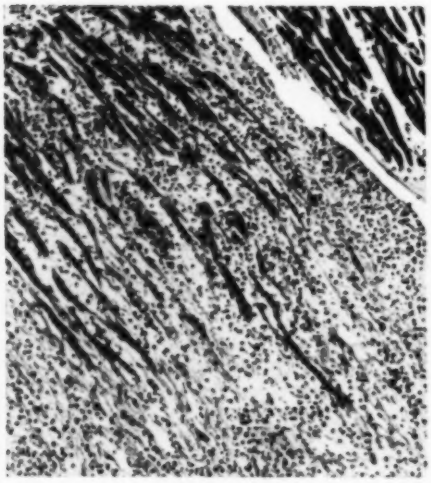

FIG. 4

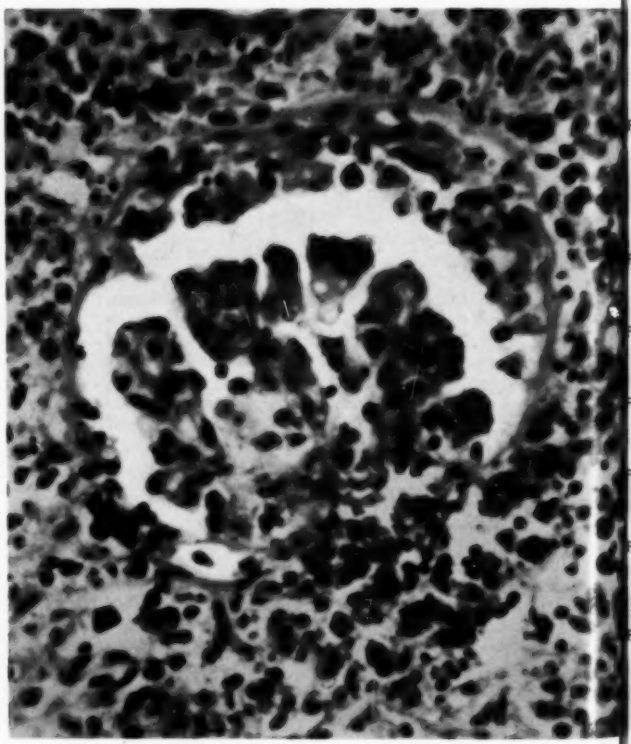

FIG. 5

FIG 6

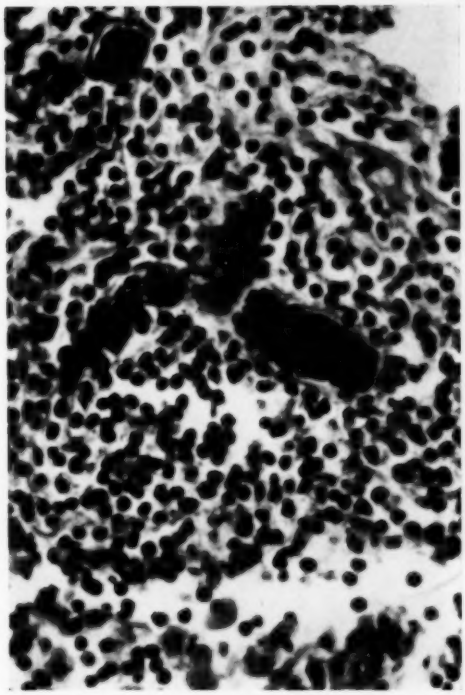

FIG. 7

FIG. 2.—Case 4, skin × 95. The corium is infiltrated by tightly packed hyperchromatic cells of uniform size.

Except where otherwise stated, all photomicrographs are from sections stained by haematoxylin and eosin.

FIG. 3.—Case 1, thyroid × 185, showing replacement of parenchyma and neoplastic cells in between surviving acini.

FIG. 4.—Case 4, heart × 90. Infiltration in between myocardial fibres and myocardial necrosis.

FIG. 5.—Case 1, kidney × 450. Tumour cells are present in the glomerular tuft and capsule and in the periglomerular tissue.

FIG. 6.—Case 4, lung × 70. Neoplastic cells surround small vessels and bronchioles and are present in alveolar walls.

FIG. 7.—Case 4, lung × 240, showing peribronchiolar infiltration. At the bottom right neoplastic cells are lying free within alveoli.

FIG. 8.—Case 2, brain × 270. Distension of Virchow-Robin space by neoplastic cells.

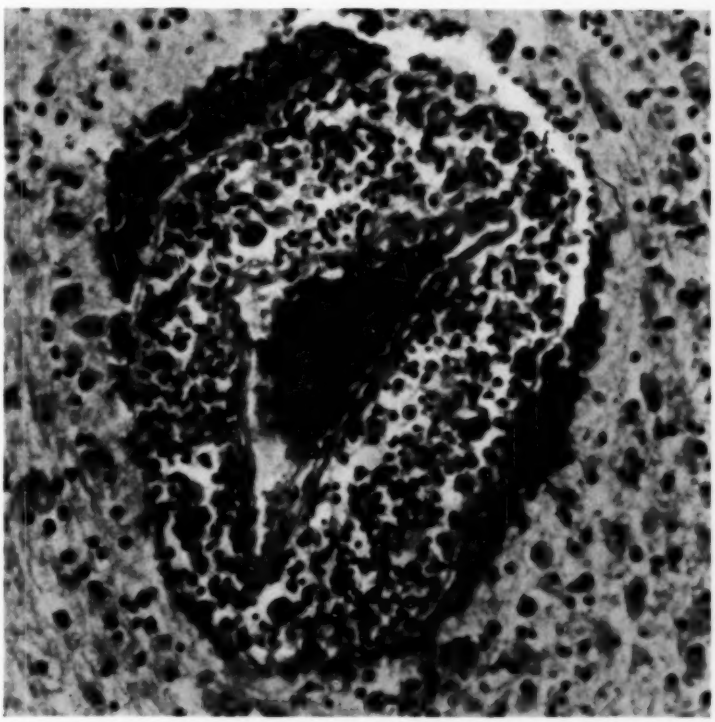

FIG. 8

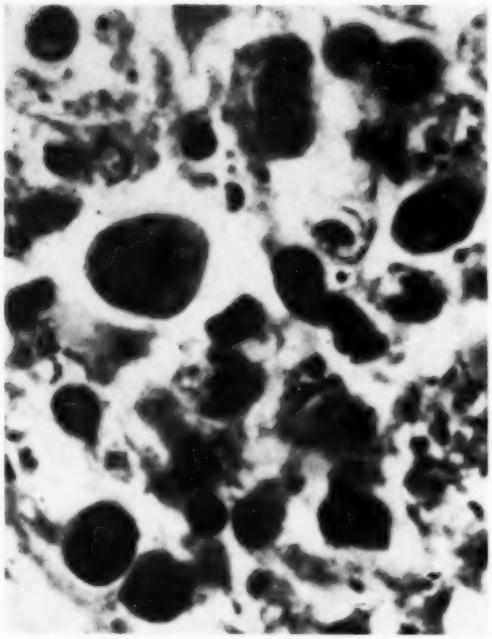

FIG. 9

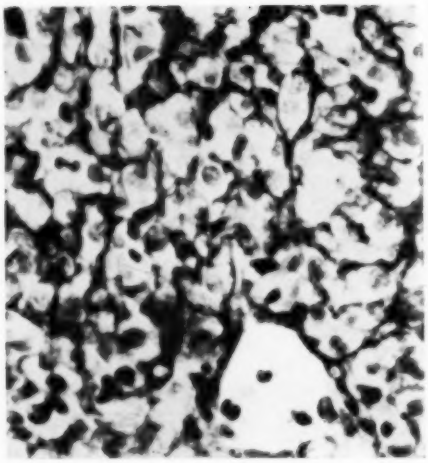

FIG. 10

FIG. 9.—Case 2, brain × 680. The infiltrate is here polymorphic and includes multinucleate cells.

FIG. 10.—Case 3, nasal biopsy stained to show reticulin fibres, × 500. A delicate reticulin meshwork encircles groups of cells.

Similar neoplastic tissue is present in slides from the macroscopic lesions of other organs, as indicated in Table I, in Cases 1, 2, and 4. In Case 3, examination of slides from eight blocks from the viscera and lymph nodes fails to reveal any neoplastic infiltration. The pattern of the infiltrates is similar in each case. Under low-power magnification the areas are pale and ill-defined. In the centre the cells are tightly packed (Fig. 2), without alveolar or other pattern, but towards the edge groups of cells are irregularly placed between the parenchymal structures. Thus in the thyroid (Fig. 3) the tumour cells extend between and around surviving acini, in the heart (Fig. 4) between surviving myocardial fibres, in the kidney (Fig. 5) into and around glomeruli. The picture in the lungs (Figs. 6 and 7) is that of diffuse perivascular and peribronchial infiltration, with extension of neoplastic cells into and often through the alveolar walls. In the liver, the portal tracts are mainly affected, with infiltration also between columns of parenchymal cells and around the central veins. Of particular interest are the cerebral metastases; from the main neoplastic masses the cellular infiltration extends outwards as a perivascular growth, causing great distension of the Virchow-Robin spaces (Fig. 8). Thence tumour cells have penetrated into the substance of the brain to form daughter tumours at a distance from the main centre. Unfortunately, the lymph nodes available show only advanced involvement, with replacement of much of the gland architecture by neoplasm: as in other sites necrosis in the centre of the infiltrates is common.

The cytology of the various infiltrates is remarkably characteristic, both from case to case and organ to organ, though the degree of polymorphism varies. The cells have medium-sized, round, oval, or lobed nuclei with a moderately dense chromatin network and a well-defined nuclear membrane. They have little cytoplasm. Sometimes the cells have a more vesicular nucleus and multinucleate forms occasionally occur (Fig. 9). Mitotic figures are easily found. Evidence of lymphatic permeation is absent and neoplastic cells are not seen within blood vessels. In some of the affected areas there is a moderate degree of fibrosis with patchy groups of lymphocytes and plasma cells at the periphery of the tumour

masses. Reticulin stains (Fig. 10) show a fine meshwork of fibres encircling groups and pockets of three or four tumour cells. No necrotizing vascular lesions or focal granulomas of the kind common in Wegener's granulomatosis are present.

Discussion

In the abundant literature on nasal granulomas which do not heal there are at least 32 cases similar to those in this paper. Most were called granuloma gangrenescens. Twenty of these, confirmed by necropsy, are summarized in Table I. Five others have been excluded from the table for various reasons. Thus, for example, there was a co-existent lymphosarcoma of the stomach in the cases reported by Schütz (Case 1, 1938) and Leroux-Robert (1957): in that described by Knapp (1949) a vaginal sarcoma was probably the primary: in Greifenstein's (1937) case the nasal lesion was preceded by mycosis fungoides in the skin: Kowalczykowa and Sokolowski (1955) were not certain that their case was neoplastic. In the seven other cases sarcomatous tissue was found in biopsy from the nasal and/or palatal region of patients still alive at the time of the report (Hesse, 1941; Pirodda and Guenzi, 1950; Eigler, 1951; Vilanova and Pinol, 1954; Guns, Marquet, and Gillain, 1956; and Hunger, 1956).

The neoplastic and malignant character of this lesion is certainly suggested by the clinical sequence of initial lesion, latent period, metastases, and fatal end. The histological picture is that of malignancy. It has from time to time been suggested that granuloma gangrenescens is not a neoplasm from the beginning but is a neoplastic transformation of classical malignant granuloma. One recent case (Hunger, 1956) in particular suggests this: in this patient, nasal ulceration continued for 15 years before malignant tissue was demonstrated and metastases developed. Furthermore, one of Woods' early cases died from sarcoma several years after the initial report (Woods, 1921). On the other hand, the average duration of the fatal cases summarized in the present report was just under one year, and, in several, malignant tissue was demonstrated in the nose at an early stage. It, therefore, appears likely that the condition is indeed neoplastic, and is so from the beginning.

TABLE II
SUMMARIZED FEATURES OF 24 NECROPSIED CASES

Sex:	
Males	14
Females	10
Age:	
Range	17–62
Mean	37
Course:	
Average	10 months
Initial Lesion:	
Nose	15
Palate	5
Both	4
Metastases	17
Lymph nodes	13
Liver	9
Skin	7
Lung	7
Kidney	6
Spleen	6
Heart	4
Ileum	4
Brain	3
Adrenal	3
Voluntary muscle	3

The naming of this neoplasm has varied with fashion, and no doubt also with the quality of the histological technique, but it is hardly justifiable to state that the lymphosarcoma described by Kanas (1936) or the sarcoma described by Hall (1934) and by Baker, Thompson, and Arnold (1958) were misdiagnoses, even though the clinical picture was similar to the cases described as reticulum-cell sarcoma by Lopes de Faria, Cutin, Morgante, and Ferri (1957) and others. My own cases certainly showed none of the classical features of Hodgkin's disease, and, although the bone marrow was not examined in any, the absence of haematological abnormality during life and of atypical cells in the blood vessels in tissue sections supports the view that the condition is not a leukaemia. The cytological characteristics in all the sections examined conform to the majority opinion that the condition is a sarcoma of the reticulo-endothelial system, the "*retothelsarcom*" of German authors, and the close resemblance of the cerebral lesions in Case 2 to the cases of microgliomatosis described by Russell, Marshall, and Smith (1948) supports this view. Following Russell's example, I have not used the term reticulum-cell sarcoma, preferring to reserve this term for the rare tumour that gives evidence of its ability to form reticulin, a feature that was not seen in any of my cases. Tumours of the reticulo-endothelial system, apart from those which simulate non-healing granuloma, are apparently rare in the nose, only two having been mentioned by Devine in his exhaustive review of neoplasms in the upper air passages reported from 1953 to 1956. Nevertheless, it seems reasonable to accept the view that the granuloma gangrenescens of the earlier writers is in fact a reticulo-endothelial sarcoma and, in most cases at least, is a neoplasm from the beginning and not a complication of a local pre-existing granuloma.

I am indebted Dr. W. Stewart, Dr. J. S. Faulds, and Dr. A. J. Wort for necropsy reports; to Dr. W. Ross for permission to publish Cases 1 and 2; to Mr. J. D. K. Dawes for permission to publish Case 4, and to Mr. J. W. Corkhill for the illustrations. It is a pleasure to acknowledge the assistance and encouragement given by Professor A. C. Lendrum, Professor J. B. Duguid, and Dr. W. W. Park.

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CHROMOBLASTOMYCOSIS SIMULATING RHINOSPORIDIOSIS IN A PATIENT FROM CEYLON

BY

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From Charing Cross Hospital and Medical School, London

(RECEIVED FOR PUBLICATION JULY 20, 1959)

A case of chromoblastomycosis confined to the mucous membrane of one side of the nasal septum is reported. The organism was not cultivated, but its characteristics in histological preparations were typical of those of the organisms in sections of cutaneous lesions known to be caused by *Phialophora pedrosoi* and related fungi. The diagnosis is considered to have been justified in spite of the great rarity of mucosal involvement in chromoblastomycosis and of the complete absence of lesions in the skin.

The patient was a Sinhalese student working in London. He had first noticed the lesion before he left Ceylon, but the symptoms of nasal obstruction and bleeding were not sufficient to make him seek medical advice until two years later. If it is correct to assume that he contracted the infection in Ceylon his case is only the second on record in which there has been reason to suggest that Ceylon has been the geographical source of chromoblastomycosis.

The lesion was excised and its site cauterized. There has been no sign of recurrence of the infection during the two years that have passed since the operation.

The chromoblastomycoses are infections caused by naturally pigmented (" dematiaceous ") fungi: because of their pigmentation these organisms appear as yellowish-brown elements which are readily seen microscopically, even in unstained preparations of infected material, including histological sections. There are two clinicopathological types of chromoblastomycosis, cutaneous and cerebral; several species of the pigmented fungi have been isolated from cases of these infections.

The main pathogenic species are *Phialophora pedrosoi*, *Phialophora verrucosa*, *Phialophora compacta*, and *Cladosporium trichoides*; of these, *Phialophora pedrosoi* (which is also known by the synonyms *Hormodendrum pedrosoi* and *Fonsecaea pedrosoi*) is by far the commonest. *Cladosporium trichoides* has so far only been identified in lesions of the central nervous system. In cutaneous chromoblastomycosis the organisms occur in the tissues in the form of rounded, spore-like structures (chlamydospores), which have a distinct cell wall and are often septate. Germinating chlamydospores with a short sprout of promycelium are seen very occasionally, but true mycelium formation does not occur in the lesions in the skin. In contrast, mycelium is characteristically abundant in the lesions of cerebral chromoblastomycosis.

Cerebral Chromoblastomycosis

Cerebral chromoblastomycosis (cerebral cladosporiosis) is an exceptionally rare infection which has been recognized only comparatively recently. It has been mortal in 14 of the 15 recorded cases. It is not usually associated with skin involvement, and in further contrast with cutaneous chromoblastomycosis its occurrence is not restricted to hot climates. The cases which have been recognized in temperate lands include one case in Britain (Symmers, 1960).

Cutaneous Chromoblastomycosis

Cutaneous chromoblastomycosis is ordinarily met with only in tropical or subtropical lands, although a small number of autochthonous cases has been recorded in the colder lands of north-eastern Europe. In most cases it is confined to one limb, usually a leg, and it is characterized by the slow development of nodular or warty, granulomatous masses which, during a period of many years, extend to cover a considerable part of the affected limb. The lesions tend to bleed easily and are prone to secondary bacterial infection. Involvement of the lymphatics in the granulomatous process eventually obstructs the lymph flow, and this may cause a lymphoedematous

elephantiasis. The infection very rarely extends from the skin into underlying structures or to the lymph nodes, and if lymphadenitis develops it is virtually always the result of secondary bacterial infection.

Chromoblastomycotic lesions in other parts of the body, the result of spread through the blood stream, have been recognized only in most exceptional cases of the cutaneous disease. In the case of Carrión and Koppisch (1933) the skin infection was on one leg and there were metastatic lesions in the subcutaneous tissue of the opposite leg and of one arm. In the case of Fukushiro, Kagawa, Nishiyama, and Takahashi (1957) multiple brain abscesses developed as a complication of infection of the skin of the face; *Phialophora pedrosoi* was isolated from the cerebral and cutaneous lesions. This is the only case of cerebral infection

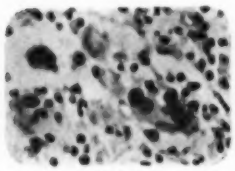

FIG. 1

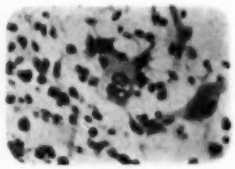

FIG. 2

FIG. 1.—A cluster of chlamydospores in a multinucleated giant cell in the right-hand half of the field; septation is clearly seen in the organism nearest to the centre of the picture. Single chlamydospores are present in two other giant cells. Note how the natural golden-brown pigment of the organisms distinctly underlies and alters the colour associated with the positive Schiff reaction. Periodic-acid-Schiff reaction; Mayer's haemalum. ×220. (Kodachrome)

FIG. 2.—Multinucleated giant cell containing a cluster of brown chlamydospores. Mayer's haemalum and eosin. ×220. (Kodachrome)

occurring as a complication of cutaneous chromoblastomycosis that has been reported; permission was not available to examine any part of the body *post mortem* except the brain, and it was therefore impossible to tell whether lesions were present elsewhere. Visceral lesions have not been recorded in any other case of cutaneous chromoblastomycosis. It should be stressed that cutaneous chromoblastomycosis in fact is not ordinarily a direct hazard to the patient's life, although considerable disability may eventually result.

Cutaneous chromoblastomycosis was first recognized in Brazil by Pedroso in 1911, but he did not publish his observations until nine years later (Pedroso and Gomes, 1920). The disease has proved to be most prevalent in the territories around the Caribbean Sea, and particularly in Puerto Rico, where it has been extensively studied by Carrión (1950), to whose work much of what is known about its manifestations and mycology is due. Cases have been recognized in most countries in the Americas, and are not very uncommon in parts of South Africa and in Madagascar and Queensland. Occasional cases have been reported from parts of China, India, Japan, the East Indies, and parts of North, East, and West Africa.

Only a very small number of cases has been reported in which the infection was contracted in Europe. The first of these was diagnosed in 1928 in Leningrad by Tschernjawski (1929): the patient, a Polish woman, had probably acquired the infection many years before when she fell from her horse into some scrub near Wilno (now Vilnius, Lithuania). Meriin (1930) gave the name *Hormodendrum rossicum* to the organism which he isolated from the lesion in Tschernjawski's case: this species, however, may well have been identical with *Phialophora pedrosoi* (Sonck, 1959b). By 1938, 14 cases had been recorded in Russia (Prokoptchouk, 1938), although it seems to be likely that the infection was not acquired in European parts of the Soviet Union in all these cases. Since then occasional further cases have been reported from the Soviet Union. The only autochthonous European cases to be recognized outside Russia were all diagnosed in Finland by Sonck (four cases—1954, 1959a, 1959b) and Järvi (one case, cited by Sonck, 1959a). *Phialophora pedrosoi* was isolated in four of these five Finnish cases; no growth was obtained in the fifth case.

The first case of cutaneous chromoblastomycosis to be diagnosed in Britain was recorded by Crow and Riddell (1954): the patient had come from Jamaica. I know of only one other case which has been recognized in Britain; this patient also came from Jamaica. His biopsy specimen was the source of the photomicrographs illustrated in Figs. 36 and 55 in an earlier publication (Symmers, 1958). *Phialophora pedrosoi* was isolated from both these cases.

Cutaneous chromoblastomycosis appears to originate with the direct inoculation of infective material into the skin as a result of minor penetrating injuries, abrasions, or the like. It is significant that the incidence of the disease is mainly in workers on the land who customarily go barefoot, and that the first lesions are usually on the foot. *Phialophora verrucosa*, one of the rarer fungi of cutaneous chromoblastomycosis, has been found in wood pulp (Martin, 1938), and there can be little doubt that the other organisms will also prove to occur as free-living saprophytes.

Involvement of the mucous membranes, as in the case reported below, is an exceptionally rare manifestation of chromoblastomycosis. Isolated instances of lesions of the conjunctiva, nose, and larynx have been described (Lacaz, 1956). There was no disease of the skin in the case to be described. The diagnosis was made in London, and the case is therefore one of the very small number of cases of this infection which have been seen in Britain. The patient was a student from Ceylon; his symptoms began before he left his home in Colombo, and there seems therefore to be no doubt that the disease originated in that country. Only one other case has been reported in which chromoblastomycosis was believed to have been contracted in Ceylon. The patient was a Canadian airman who had served there for two years during the second world war: his lesion appeared before he left the island, and the diagnosis was made in Canada about four years later (Burns, 1950). The possibility that the Canadian patient acquired the infection in North America, or during calls ashore in South Africa and India on the way to Ceylon, cannot be excluded.

I am indebted to Professor G. R. Cooray, of the University of Ceylon, for the information that chromoblastomycosis has not to his knowledge been recognized in his country on other occasions. It is relevant that only three cases of presumed chromoblastomycosis have been recorded in India (Andleigh, 1953; Thomas, Job, and Hadley, 1957; Kakoti and Dey, 1957): I am indebted to Lieutenant-Colonel M. L. Ahuja, Medical Adviser to the High Commissioner for India in London, to Lieutenant-Colonel B. K. Sheorey, also of India House, London, and to Professor R. N. Chaudhuri, Director of the School of Tropical Medicine in Calcutta, for this information, and to the Administrator of King Edward Medical College in Lahore for the information that no case of chromoblastomycosis is known to have occurred in Pakistan.

Case Report

A young Sinhalese man, studying in London, came to hospital because of troublesome bleeding from a small mass which was partly obstructing the airway in one nostril. He had noticed the lesion first some two years earlier, a few weeks before he left his home in Colombo to come to England. He had never been out of Ceylon before then. At first there were no symptoms, and he noticed the little mass—which could be seen through the nostril—quite by accident. It grew very slowly, and it was not until after about nine months that he began to have a sensation of pressure and obstruction in the nose. Some dry crusting formed around the lesion, and in gently attempting to clear the crusts away with a probe he caused free bleeding on a number of occasions. He became accustomed to the slight discomfort, and apart from a tendency to bleeding if he blew his nose too forcefully he had no reason to feel particularly concerned about it. On some occasions, however, the bleeding was difficult to stop, and his nose filled with blood clot which he was afraid might become infected: he therefore felt it advisable to have something done about the condition.

Examination showed a fairly soft, dusky red, sessile mass projecting from the nasal septum and filling the airway between it and the anterior part of the lowest turbinate. The surface of the lesion was smooth and sparsely patterned with minute, rounded flecks reminiscent of the similar appearance of the lesions of rhinosporidiosis. As the patient had come from Ceylon, where rhinosporidiosis is known to occur, this diagnosis was made clinically with considerable confidence. The lesion was dissected out with little difficulty, and the site was cauterized with the intention of destroying any remaining organisms. No other lesions were found. No attempt was made to put up cultures; the whole of the excised tissue was fixed in formol-saline.

The specimen consisted of about a dozen pieces of soft, vascular tissue which occupied in all a volume of just over 3 c.cm. Sections showed no sign whatever of rhinosporidiosis, and to confirm this the whole of the tissue was examined histologically in step sections. Instead of the expected sporangia and spores of *Rhinosporidium* almost every high-power field contained dark golden-brown, rounded bodies, each about 10 μ in diameter, which were present singly or in clusters of up to six or eight (see figures). Many of these bodies were divided by one or two septa; no hyphae were found. The appearances were typical of the chlamydospores (so-called "sclerotic bodies") of the fungi which cause cutaneous chromoblastomycosis, as was readily confirmed on comparing them with the organisms in histological preparations from several known cases of *Phialophora pedrosoi* infection. It may be noted that the morphological appearances of the chlamydospores in infected tissues do not enable the various species of fungi which cause chromoblastomycosis to be distinguished from one another; they can be distinguished only by their cultural characteristics.

As in cutaneous chromoblastomycosis, the tissue reaction in this patient's nasal lesion was a complex type of chronic granuloma, in which suppurating pseudotubercles were the most characteristic feature, with polymorphonuclear leucocytes, lymphocytes, and a few plasma cells in the loose-textured intervening granulation tissue. The organisms were present both in multinucleated giant cells in the pseudotubercles and free in the purulent exudate of the microabscesses.

The histological diagnosis of chromoblastomycosis led to a belated, and unsuccessful, attempt to isolate the organism from the nose. Clinical and radiological examination showed no evidence of any abnormality

of the nose, sinuses, ears, mouth, throat, and eyes. The patient's skin was healthy, and radiographs of his chest were normal. The operation site healed rapidly.

When the patient was last seen two years after the operation, there was no sign of any recurrence of the infection.

Comment

In spite of the unusual location of the lesion, of the failure to confirm the diagnosis by cultivation of the causative organism, and of the apparent origin of the disease in a country where this infection is not known with certainty to occur, there seems to be no reason to doubt that the case was one of chromoblastomycosis. The occurrence of chromoblastomycosis in Ceylon would not be surprising. It is, however, remarkable that the lesion should be on a mucous membrane in the first case to be recognized in a patient from that country: in other parts of the world the infection is presumed to be acquired by the direct implantation of infected material—probably soil—into the skin, the legs being particularly liable to infection because they are so frequently subjected to wounds contaminated by soil; one can only speculate on the source and mode of infection of the nasal mucosa in the case described.

In view of the original clinical diagnosis of rhinosporidiosis in this case it is important to stress that the histological distinction between the organism present in the tissues and *Rhinosporidium* is absolute. First, *Rhinosporidium* is not pigmented. Secondly, although the largest spores of *Rhinosporidium* are comparable in size with the chlamydospores of the fungi which cause chromoblastomycosis they differ from the latter in being more uniformly spherical, in having a much less distinct cell wall, and in the absence of septa. Finally, free spores of *Rhinosporidium* would not be expected in the tissue in the absence of the typical sporangia: the sporangia, which range in diameter from 100 to 350 μ or more and contain great numbers of spores at various stages in development, are very conspicuous structures and their presence could not be overlooked.

This case is a further example of the need to be prepared nowadays to meet with strange diseases in countries like Britain which have become more cosmopolitan than was possible when international travel was a greater and rarer undertaking than it is to-day. It has become more important than ever to have an accurate and complete history of the geographical background of every patient, and a complementary knowledge of the diseases to which residence or travel in other countries may have exposed him. This is as true of, for instance, British folk who may have been oversea as it is of those who come from abroad to visit or work in this country.

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Addendum.

In July, 1960, while this paper was going to press, Professor Cooray told me that several cases of cutaneous chromoblastomycosis have been recognized in Ceylon since June, 1959, when he prepared the information mentioned in the text on page 289. The patients have all been residents of Ceylon. The clinical and histological appearances were typical. It has not yet been possible for any of the cases to be investigated mycologically.

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POLYARTERITIS NODOSA AND CONGENITAL PYLORIC HYPERTROPHY IN A 3-MONTH-OLD INFANT

BY

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A case of polyarteritis nodosa in the first year of life with the clinical picture dominated by pyrexia, an initial upper respiratory element, episodes of tachycardia, and sudden death three weeks after the onset is described. The necropsy showed evidence of polyarteritis nodosa, myocarditis, and valvulitis as well as congenital pyloric hypertrophy. Clinical manifestations of the latter had preceded the final illness and the two diseases were not thought to be related.

The previous 19 cases reported in the first year of life are reviewed, and support is given for the concept that at this age a rash, pyrexia, upper respiratory manifestations, and progressive heart failure or sudden death are characteristic clinical manifestations and that the heart is often the site of the major lesions at necropsy.

The possible role of hypersensitivity is discussed. There was no evidence for it in the present case.

Polyarteritis nodosa is uncommon in infancy; references to 19 cases diagnosed as such in the first year of life have been found; there are a few others we have not been able to check (the cases of Rüger, 1944, and Wright, 1948). Of those checked all except the ones reported by De Pascale (1955) and Legros (1944) have proved fatal. These authors reported cases in which the main manifestations of the disease were in the skin, and it is possible that they were not dealing with examples of true polyarteritis nodosa but rather that group described as "allergic" vasculitis by McCombs, Patterson, and McMahon (1956) in which recovery is common. Many of the fatal cases had features indicating involvement of the skin, upper respiratory tract, and heart during life and maximal lesions in the coronary arteries at necropsy. In the infant reported in this paper such features were preceded by clinical symptoms of congenital pyloric hypertrophy which was also found at necropsy. Furthermore, a diffuse myocarditis and valvulitis similar to that of severe acute rheumatism formed part of the pathological picture.

Case Report

C.Q., a boy, had weighed 9 lb. 7 oz. at birth, the pregnancy and labour being normal. The father had had pulmonary tuberculosis which was reported cured in 1946. There was no other family history of illness and none of allergy. The infant was breast fed and at the age of 9 weeks was admitted to hospital with a history of constipation and increasing vomiting after feeds for two weeks; in the preceding four days every feed had been returned. A provisional diagnosis of hypertrophic pyloric stenosis was made. On examination the baby was well nourished and hydrated. There was visible peristalsis, but no pyloric tumour was palpated and there were no other physical signs. The vomiting ceased during administration of atropine methyl nitrate which was continued for several days.

In the absence of a pyloric tumour a diagnosis of feeding difficulty was made and he was discharged home after three days. The infant remained well and continued to gain weight until the age of 12 weeks, when a cough, purulent nasal and conjunctival discharge, and bilateral cervical lymph node enlargement developed. After five days he was again admitted to hospital; pyrexia, mostly 100–103° F., swinging at times, was present during the remainder of his life, a further two and a half weeks; the liver was palpable to two fingerbreadths.

Investigations.—No pathogens were isolated from nose and throat swabs or from faeces. The cerebrospinal fluid was clear; cells 16/c.mm. (50% lymphocytes, 50% endothelial cells); protein 30 mg./100 ml., sugar 55 mg./100 ml.; culture sterile. A blood count showed Hb 70%; red cells 4.3 million; white cells 32,000 (neutrophils 85%, eosinophils 5%, lymphocytes 7%, monocytes 3%, reticulocytes 0.2%); blood urea 35 mg./100 ml. A blood culture showed a coagulase-negative staphylococcus (regarded as a contaminant). There was no agglutination with *S. typhi, paratyphi,*

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FIG. 1.—Photograph of heart. At 1, 2, and 3 aneurysms of the coronaries containing thrombus are seen in section. At 4, a branch of the coronary is cut across as it runs down the surface of the ventricle; the thickened wall causes the vessel to stand out as a cord.

and *typhi murium*. A direct Coombs test was negative. The Mantoux test at 1:100 was negative. A report on iliac marrow (Dr. H. Caplin) read:

> "... the main feature was intense granulocytosis with a preponderance of metamyelocytes and juvenile polymorphs. Nucleated red cells appeared scanty, the myeloid-erythroid ratio being approximately 30:1. The appearances were in keeping with a pronounced reaction to pyogenic infection."

Urine contained no protein or sugar; a few leucocytes and epithelial cells were found in the deposit and a culture showed coagulase-negative staphylococcus (regarded as a contaminant).

Radiographs of the chest and the whole skeleton were normal. An electrocardiogram with a rate of 180 per min. approximately and with right axis deviation was interpreted as indicative of sinus tachycardia only.

Progress and Treatment.—A single injection of crystalline penicillin, 100,000 units, was given on admission, then chloramphenicol, 250 mg. by mouth six hourly for three days; and then streptomycin, 100 mg. 12 hourly, and penicillin, 100,000 units four hourly, both intramuscularly, for a further three days. Six days after this, terramycin, 70 mg. six hourly by mouth, was given for 48 hours. None of these drugs had any effect on the pyrexia. The only other drug given was atropine methyl nitrate, 3 drops before feeds during the first five days of this second admission.

A sudaminal type of rash appeared on the upper limbs three days after admission and was present intermittently during the rest of the illness on all limbs. The liver remained palpable, the apical pulse rate was 160–180 on two occasions, the complexion was sallow but without jaundice. Feeding was satisfactory and stools were normal. Dr. Kenneth Tallerman kindly agreed to accept responsibility for the care of the baby and he was transferred to the London Hospital where he suddenly collapsed and died 60 hours after admission. In that time the laboratory findings remained unchanged; a chest film was normal and a blood culture sterile.

Necropsy Report.—The findings may be summarized as acute heart failure, myocarditis, polyarteritis nodosa, and congenital pyloric hypertrophy. Body weight 15¼ lb.

Heart (2 oz.).—Scattered nodular aneurysms of the circumflex branch of the left and terminal part of the right coronary artery were present; they had a bluish-red, projecting surface and on section contained recent thrombus (Fig. 1), the latter extending for a few centimetres on either side of the dilatation. The wall of the right coronary artery was slightly thickened (up to 0.05 cm.) proximal to the thrombus. Most of the free margin of the mitral valve and a segment (1.5 cm. long) of the tricuspid valve were slightly

FIG. 2.—Wall of left auricle showing inflammatory infiltrate almost as thick as the muscle of the wall. Verhoeff's elastic stain, × 26

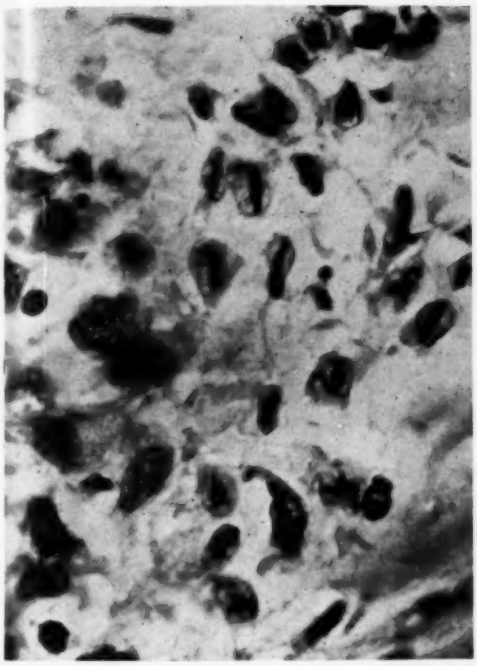

FIG. 3.—Infiltration in subendocardial region of left ventricle showing numerous Anitschkow myocytes. Haematoxylin and eosin, ×830.

FIG. 4.—Right coronary artery proximal to thrombus with considerable inflammatory thickening of the intima and focal destruction of elastic and muscle of media. Verhoeff's elastic stain, × 23.

FIG. 5.—Artery in splenic capsule with conspicuous inflammatory infiltration and focal destruction of prominent internal elastic lamella and muscle of media. Haematoxylin and eosin, × 110.

FIG. 6.—One segment of artery of Fig. 5. Mononuclear cells predominate in the infiltrate. There are a few smudges of fibrinoid material (black) just internal to the elastic lamella, especially in the concavity of the segment. Haematoxylin and eosin, × 330.

thickened; pinhead haematomas were also present in these, one at the root of the tricuspid, one near its free edge, and a third near the free edge of the mitral. The myocardium was firm and greyish brown with a slightly streaky appearance on the fresh cut surface in some areas of the left ventricle.

Other Organs.—The kidneys were congested; the lungs slightly congested. The muscle of the pyloric end of the stomach was conspicuously thickened (0.5 cm.), but there was no stenosis.

Microscopical Examination.—This was undertaken on tissues from all the cardiac chambers, mitral and tricuspid valves, coronary arteries, liver, spleen, kidneys, alimentary tract, endocrines, several lymphatic glands, the submandibular salivary gland, both lungs, cerebellum, pons, basal ganglia, and left temporal lobe.

There was an extensive inflammatory infiltration in the heart which was maximal in and around the walls of the larger coronary arteries, in the endocardium of the left auricle (Fig. 2), and at the root of the mitral valve but was also conspicuous in patches in the subendocardial region of the left ventricle and to a less extent in the right auricle and ventricle. The walls of small coronary arteries were in general spared, but the endothelium of a few veins was infiltrated. The mitral valve was infiltrated from root to free margin but the tricuspid only at the root. Histiocytes and lymphocytes were very numerous in all sites; polymorphonuclear leucocytes abundant in most sites, numerous eosinophil leucocytes in only a few sites. In some areas there were scattered fibroblasts and some delicate, newly formed collagen fibrils. In the subendocardial region of the left ventricle Anitschkow myocytes were abundant (Fig. 3) and in several areas some were large with twisted hyperchromatic nuclei reminiscent of the cells found in Aschoff bodies; an occasional histiocyte was seen in mitosis. In the myocardium the infiltrate was perivascular for the most part, but a few small groups of cells were found between muscle fibres. In some areas the connective tissue at the site of infiltration stained poorly and in a few there was slight fragmentation, but fibrinoid change was only seen in one small area in the epicardium anterior to the interventricular septum. The infiltration was thus somewhat like that of acute rheumatism, but the cellular types were even more pleomorphic than in that condition.

In the larger coronary arteries the infiltration was often associated with partial destruction of the elastic lamellae and of muscle of the media; in some the thickening of the intima was very conspicuous (Fig. 4), but in none was there fibrinoid material in the wall. At the sites of aneurysms there was great focal thinning of the media and recent thrombus in the lumen.

A similar arteritis involved one vessel in the splenic capsule (Figs. 5 and 6) and two in the capsules of the suprarenals. In the splenic one there was focal destruction of the internal elastic lamella, patches of fibrinoid internal to it, and conspicuous cellular thickening of the intima; in the suprarenal vessels the internal elastic lamellae had a fibrinoid layer closely apposed to their inner aspect, but appeared to be intact. The adventitia was conspicuously thickened by cellular infiltration, and this encroached in places into the media.

In the section of the upper lobe of the left lung focal infiltrations of the walls of large pulmonary arteries and veins mainly by polymorphonuclear leucocytes and lymphocytes, were associated with intimal intraluminal projections and focal partial destruction of the elastic of the wall in the arteries. In this and also in the section of the right upper lobe chronic inflammation of the walls of a few bronchi was present. In the kidney there was adventitial infiltration of an artery in the lower cortex.

The only other histological abnormalities were inflammation of the leptomeninges, of the capsule and periportal regions of the liver, of the sinuses of the lymph glands and around a few small veins in the thyroid.

Reasons for Diagnosis.—Destructive and inflammatory lesions of large branches of the coronary arteries and of smaller ones in the suprarenal and splenic capsules were present, the former being accompanied by aneurysm formation and thrombosis, the latter showing focal fibrinoid change. These justify a diagnosis of polyarteritis nodosa. The presence of slighter but definite lesions in pulmonary arteries and veins makes its nosological place uncertain if the classification of Zeek (1952) is used. This and the significance of the myocarditis will be considered in the discussion. The congenital pyloric hypertrophy is regarded as a coincidental and unrelated finding.

Discussion

It is convenient to summarize five of the cases reported of polyarteritis nodosa occurring in infancy, and to give very brief details of others, in order to illustrate the variety of clinical and pathological manifestations before considering the significance of the peculiar features of the present case.

Krzyszkowski (1899) described a 2½-month-old baby with an indefinite skin rash, slight oedema of the eyelids and extremities, and pyrexia. A clinical diagnosis of scarlet fever or syphilis was made and death occurred 11 days after the onset. Necropsy showed small thrombosed aneurysms of the coronary arteries which were characteristic of polyarteritis nodosa on microscopic examination. Although bronchopneumonia, enteritis, and nephritis were also described, arterial lesions were not found outside the heart.

Scott and Rotondo (1944) described a 9-month-old female in whom a rash developed after the fifth dose of sulphathiazole (0.5 g. every four hours was given for pharyngitis with pyrexia.

Pyuria and oedema of the face and limbs were later manifestations, while radiographs indicated cardiac hypertrophy and an inflammatory process at the base of the right lung. The child died suddenly five weeks after the initial symptoms at a time when clinical improvement was thought to be occurring. At necropsy, thrombosed aneurysms were present in the coronary arteries of the enlarged heart. Microscopy indicated these to be due to polyarteritis nodosa, which was also found in the kidneys, liver, and uterus.

Fager, Bigler, and Simonds (1951) reported a 4-month-old male infant who had an illness resembling virus pneumonia initially. Pyrexia was persistent despite treatment with penicillin, streptomycin, aureomycin, and sulphonamides. There was no rash, but in the terminal stages the respiratory and cardiac symptoms and signs suggested a myocarditis. At necropsy thrombosed dilated coronary arteries in the enlarged heart were recanalized and there were infarcts in spleen and kidneys ; the left renal artery was thrombosed. Microscopy indicated polyarteritis nodosa in these organs and focal myocarditis.

Adelson (1951) described a male infant in whom the illness began at the age of 15 weeks with pyrexia, slight cough, and moderate cervical gland enlargement which persisted throughout an illness of 22 days. Sensitivity to penicillin, which had been used initially, was strongly suggested by the development of a rash following its administration on two separate occasions. The final episode of screaming, gasping, and cyanosis in which death occurred was sudden and unexpected. At necropsy there was conspicuous nodular " beading," narrowed lumina, and aneurysmal dilatation of coronary arteries with thrombosis in the circumflex. The histology showed characteristic changes of polyarteritis in the heart and other organs.

Martelle (1955) did not classify his case of " coronary thrombosis in a 5-month-old infant " as an example of polyarteritis although the clinical and pathological features were similar to those of many recorded examples. The illness started at 4 months with fever, vomiting, loose stools, and a dry skin. The baby was treated with penicillin and appeared to recover satisfactorily. Two weeks later there was a two-day episode of irritability, anorexia, and vomiting without pyrexia. The baby recovered, but when 5 months old he had another episode in which sudden death occurred. At necropsy the enlarged heart showed aneurysmal dilatation and thrombosis of all the coronary arteries. Microscopical examination showed severe arteritis, involving chiefly the coronaries, but also the aorta and extensive myocarditis and endocarditis. The latter affected the left auricle but not apparently the valves ; and although distinct Aschoff bodies were not present " the grouping of some of the mononuclear cells was suggestive, as well as their individual appearance." Areas of muscle necrosis were not thought sufficient to account for the myocarditis.

Of the other cases recorded it is worth while noting that marked pulmonary arteritis was a feature in the newborn infant described by Elwood (1955) and in the case of Liban, Shamir, and Schorr (1954).

Johansmann and Zeek (1954) described a 7-day-old baby in whom scattered arterial lesions were thought to correspond to that type of polyarteritis nodosa which she refers to as the classical one and in which hypertension may be an important pathogenic factor. This example had also a thrombosed aneurysm of a ductus arteriosus and extensive infarction of the right kidney. The latter was associated with thrombosis of a branch of the renal artery but not with arteritis.

The infant described by Bohman (1954) was unique in that the illness began after pertussis prophylaxis had been given, but the course suggested that a septic process, starting in subcutaneous abscesses, was more important in pathogenesis. In Thinnes' (1924) case the only symptom was abdominal pain four hours before death and at necropsy histological evidence of polyarteritis was present in the heart and kidneys. The two cases described by Wilmer (1945) occurred in the first month of life ; both had a severe illness, one with skin lesions, and extensive lesions were found in the heart, kidneys, uterus, and other viscera. The 9-month female infant reported by Diaz-Rivera and Miller (1946) fell into the skin, upper respiratory, cardiac group, as did that of Sinclair and Nitsch (1949). In both of these sulphonamide drugs had been given, in the latter penicillin also.

The case reported by Korb (1957) had a clinical picture suggestive of nephritis and sepsis, and polyarteritis was only diagnosed on microscopic examination ; that of Rose (1957) had aneurysms of branches of the aorta as well as coronary involvement.

Munro-Faure (1959) has reported an infant with marked coronary involvement and reviewed a series which overlaps extensively with the one considered here. Her paper is called " Necrotizing Arteritis of the Coronary Vessels." She has therefore not included examples of poly-

arteritis in which the coronaries were not involved and has included two cases in which the authors, Crocker, Sobin, and Thomas (1957), considered the diagnosis of polyarteritis nodosa but were not prepared to make it. Before reading her paper we had noted the frequent involvement of the skin in life and of the coronary arteries at necropsy in this group.

In her somewhat more selected series this point comes out clearly, and we would support the contention that there is a group of cases in infants in which an arteritis is maximal in the coronaries and the illness is frequently characterized by fleeting macular skin eruptions, fever, leucocytosis, conjunctivitis, and pharyngitis, and in which death occurs either as a result of progressive heart failure or suddenly and unexpectedly. Cervical adenitis and cough were common but not quite so frequent in this group. If the features of the pyloric hypertrophy are regarded as a separate process the present case obviously illustrates many features of what might be called the upper respiratory, skin, cardiac group. Of the 18 cases discussed here, eight fall easily into this group and lesions of the coronaries were present to some extent in 16 (88%). In the 111 cases of polyarteritis recently reviewed by Rose (1957), which included mainly adult material, 48% had coronary lesions. In none of these infantile cases was there the prolonged uraemia, hypertension, or peripheral neuritis which characterize an appreciable number of adult cases.

There remain for consideration the significance of the myocarditis and valvulitis in our case and the possible role of hypersensitivity in the pathogenesis. The myocarditis was probably responsible for the episodes of tachycardia during life, and a similar correlation has been noted in other infantile cases. Extensive myocarditis, however, is uncommon in this age group, endocarditis was only noted in the case of Martelle (1955), while the valvulitis of the present case was unique.

The presence of these features suggests that our case might be classified as an example of rheumatic carditis with polyarteritis. Such have been described in the past by Neale and Whitfield (1934) and Friedberg and Gross (1934) in children and adults but not in infants. The only infant which could be so classified in addition to our own is that of Martelle (1955). Clinical manifestations of rheumatism outside the heart and the association with a streptococcal infection which have been a feature of rheumatic carditis with polyarteritis were lacking in the infantile cases. At this age, furthermore, acute rheumatism is almost unknown.

We do not therefore wish to press this point nor the interwoven one of the possibility of a hypersensitive reaction in the pathogenesis. This would involve a detailed discussion of present-day views on the role of hypersensitivity in polyarteritis which was originally emphasized so clearly by Rich (1947) and more sharply defined in Zeek's (1952) concept of "hypersensitivity angeiitis." Our case does not fit into this category and the concept has not been confirmed in the survey of Rose and Spencer (1957). And although the present example yields no clear evidence of a hypersensitive reaction to any of the drugs given, one is left with the impression that the morphological changes are more easily explained in terms of such a process than in any other way. That antibodies can form in the early months of life is indicated by the investigations of Sako (1947) and Sako, Treuting, Witt, and Nichamin (1945), and the case of Adelson (1951) suggests strongly that anaphylactic hypersensitivity can develop actively at this period. It is possible that in these early months there may be considerable variation in the speed and degree of loss of the immunological tolerance of foetal life and occasional unusual manifestations of reaction to antigenic stimulation.

We are indebted to Dr. Eric Frankel and Dr. Kenneth Tallerman for allowing us to describe a case under their care, and to Professor Dorothy Russell for advice about the pathology.

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THYMOLIPOMA: A BENIGN TUMOUR OF THE THYMUS GLAND

BY

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An example of a thymolipoma is described and the literature reviewed.

Thymolipoma (or lipothymoma) is a benign tumour of the thymus gland consisting of fat and thymic tissue. The only symptoms it produces, if any, are the results of pressure on surrounding structures. It is not a common tumour. No example was found in the 67 thymic neoplasms studied by Thomson and Thackray (1957). Seventeen isolated reports have been found in the literature (Table I) and one more is presented here.

Case Report

The patient was an apparently healthy boy aged 7 years. He was referred to hospital because his general practitioner found persistent signs in the right lung after a mild respiratory infection. Radiographs showed an unusual rounded shadow to the right of, and continuous with, the mediastinum. Investigations were otherwise negative.

TABLE I

SUMMARY OF CASE REPORTS

Case No. and Author	Age of Patient (Years)	Sex	Weight of Tumour (g.)	Symptoms
(1) Lange (1916) ..	58	F	1,600	Dyspnoea
(2) Yamanoi (1921)	42	F	Not stated	Asymptomatic
(3) Andrus and Foot (1937) ..	13	M	2,235	Dyspnoea
(4) Hall (1949) ..	47	M	1,100	Asymptomatic
(5) Schanher and Hodge (1949)	35	F	625	Dyspnoea
(6) Bariéty and Coury (1950) ..	40	F	350	,,
(7) Fontaine *et al.* (1951) ..	24	F	750	,,
(8) Bigelow and Ehler (1952)	10	F	170	Cough
(9) Gross (1953) ..	14	M	Not stated	Asymptomatic
(10) Rubin and Mishkin (1954)	19	F	750	Dyspnoea
(11) Guilfoil and Murray (1955)	22	M	540	Cough
(12) Dunn and Frkovich (1956)	47	M	6,000	Dyspnoea
(13) Falor and Ferro (1956) ..	24	F	2,268	Asymptomatic
(14) Mackay-Dick *et al.* (1956) ..	36	M	1,142	,,
(15) Ringertz and Lidholm (1956)	28	M	Not stated	,,
(16) Unver (1957) ..	26	M	,,	,,
(17) Andritsakis and Sommers (1959)	Not stated		54	,,
(18) Present case (1960) ..	7	M	154	,,

After a few months' observation it was decided to explore the chest. A large fleshy mass was found attached to the right side of the pericardium extending from the diaphragm up to the superior vena cava, and appeared to be an enlarged right lobe of thymus. The thymic vein was large and drained into the vena cava. The whole gland was successfully dissected from the major vessels, phrenic nerve, and other structures, and the chest closed. Post-operative progress was good and the patient is now clinically and radiologically satisfactory.

Operation Specimen

The tissue weighed in all 154 g. The normal range of thymic weight for a child of this age is 15–55 g. (Boyd, 1932).

The material consisted of two irregularly shaped pieces, the larger $11.5 \times 9 \times 4.25$ cm., representing the right lobe of the thymus. The smaller, which constituted the left lobe, measured $6.25 \times 3 \times 2$ cm. and appeared to be normal tissue.

The larger specimen was pale pink on section. It was divided by very fine trabeculae into a few lobules and was spattered with a few petechial haemorrhages which were probably due to operative trauma. There were no cystic areas. It was slightly firmer than an ordinary lipoma.

Histology

The specimen from the left side is normal thymus.

The tumour from the right consists of intermingled lipomatous and thymic tissue. The proportions of the two vary from area to area, but otherwise sections from 10 different parts all show essentially the same picture. About three-eighths of the total specimen is fat and the remainder thymic tissue which does not show the normal cortical and medullary arrangement, although Hassall's corpuscles are quite numerous. No lymphoid follicles are present and there is no evidence of malignancy. At the border between thymic and fatty zones there is a thin condensation of fibrous tissue in some places, but in other areas the lymphocytes and epithelial cells fray out into the fat (Figs. 1 and 2). The fat is of well-differentiated adult type. A blood supply of normal vessels intersects the specimen.

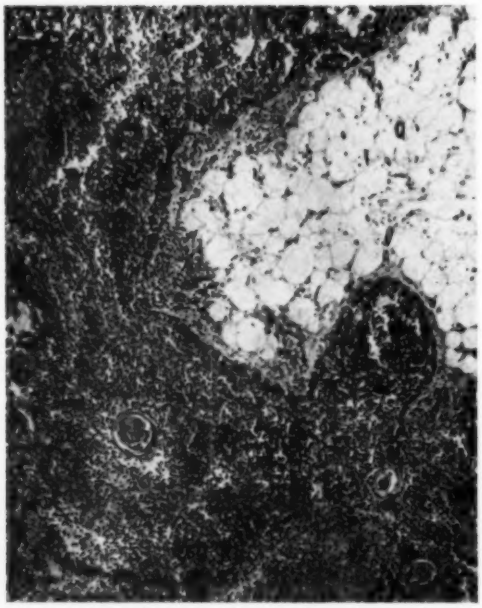

FIG. 1.—Fatty and thymic tissue, the latter showing Hassall's corpuscles (haematoxylin and eosin, × 77).

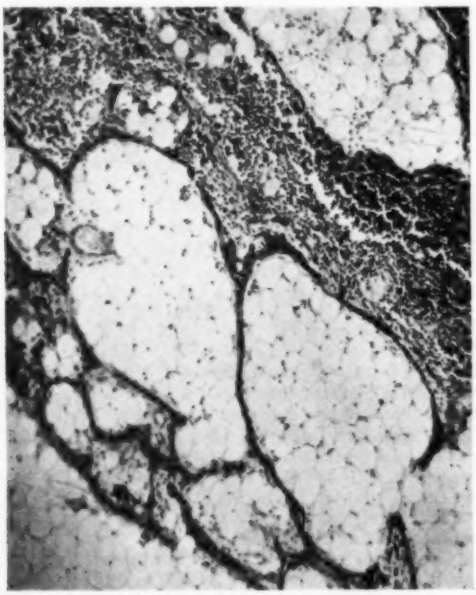

FIG. 2.—Intermingled fatty and thymic tissue (haematoxylin and eosin, ×77).

Discussion

In pseudolipomatous hypertrophy of the pancreas (Beresford and Owen, 1957) there is a marked increase in fat, but the exocrine tissue is greatly reduced. In thymolipoma, however, the thymic tissue is increased in amount, as well as the fat. Some specimens have reached very large sizes (6,000 g. in the case of Dunn and Frkovich, 1956), and it would seem reasonable to regard them as neoplasms rather than hyperplasias, especially as the thymic tissue is not arranged in quite its normal orderly way. Hall (1949) thought that these tumours are probably best regarded as mixed tumours, and this view has been supported by others (Bigelow and Ehler, 1952; Guilfoil and Murray, 1955).

Growth appears to be very slow, and in one case (Schanher and Hodge, 1949) the tumour was known to be present for at least 10 years. It may well be that the origin always dates back to childhood. Except for the case of Andrus and Foot (1937), the biggest tumours have been in adults, and from the descriptions given one has the impression that fat is the more predominant in the older cases. Dunn and Frkovich suggest that the fat originates in an involuting hyperplastic gland, and they reject the term " thymolipoma " because they consider that if allowed to express their natural course these tumours will grow as lipomas whilst the glandular elements lose their proliferative tendency.

Rubin and Mishkin (1954) go so far as to suggest that all mediastinal lipomas originate in the thymus. This extreme view seems to us improbable, but further observations are needed to clarify the issues. It is interesting to note that five of the reported thymolipomas have occurred in one lobe only of the gland.

Our thanks are due to Professor R. A. Willis for encouragement and advice, to Mr. J. E. Wilson for permission to use the clinical notes, and to Mr. G. Tulloch for technical and photographic assistance.

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POLYCYTHAEMIA VERA AND CHRISTMAS DISEASE WITH SLIGHTLY PROLONGED PROTHROMBIN TIME

BY

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A family is reported in which Christmas disease in one member and polycythaemia in another were associated with prolongations of the one-stage prothrombin time of the factor-VII deficiency type.

Changes in the prothrombin time were present in the siblings of the case of Christmas disease and in a number of unrelated cases of polycythaemia vera.

The use of " mixing procedures " did not reveal any significant mutual correction of the changes in prothrombin time.

de Vries, Kettenborg, and van der Pol (1955) described patients in whom serious abnormalities of blood coagulation were associated with slightly prolonged " one-stage " prothrombin times. They state that " generally not sufficient attention has been or is being paid to an apparently slight prolonging of the one-stage prothrombin time in clinical laboratories." Since then, a number of examples of combined clotting abnormalities of this type have been reported. In the cases of Bell and Alton (1955), Stein and Abrahams (1956), and Nour-Eldin and Wilkinson (1959), the serious coagulation abnormality is Christmas disease, while Constandoulakis (1958) describes a family showing true haemophilia also associated with prolonged prothrombin times in some of the members.

In these cases the prolonged prothrombin times have been due to a change in the patients' sera of the " factor-VII deficiency " pattern ; the addition of 10% of normal serum rich in factor VII to the patients' plasma restores the prothrombin times to normal ; the patients' sera are not so effective as normal sera and plasma in correcting the prolonged prothrombin times of plasmas from patients receiving anticoagulant drugs of the dicoumarol type. Prolongations of the " one-stage " times due to deficiency in factor V and in prothrombin itself have been excluded by the appropriate tests. In these studies, a similar prolongation of the prothrombin time has been noted as an isolated change in the blood of the clinically unaffected relatives of patients with severe bleeding tendencies, some of the latter showing the combined defect. The changes are usually slight, the prothrombin times exceeding those of normal control plasmas by only a few seconds. It appears that the alterations in factor-VII activity are an associated inherited change.

Patients with polycythaemia vera frequently have an increased tendency to bruise or bleed ; however, the various blood coagulation investigations often give normal results and there are no constant changes. Björkman (1948) found decreased amounts of fibrinogen in the blood of patients with polycythaemia. In addition, his Case 1 showed a prolonged prothrombin time.

The purpose of the present paper is to describe the findings in a family in whom Christmas disease in one member and polycythaemia vera in another were associated with minor prolongations of the prothrombin time. The occurrence of differing blood dyscrasias within this family prompted study of unrelated cases of polycythaemia vera and allied disorders.

Family History

The family tree as far as can be traced is shown in Fig. 1, the tendency to bleeding affecting only male members and showing sex-linked inheritance.

The S Family

Charles S. died aged 68. He had always bruised easily and had had occasional nose bleeds. When aged 65 he had an operation for perforated peptic ulcer. In December, 1957, he began to lose strength and complain of giddiness. The spleen was not

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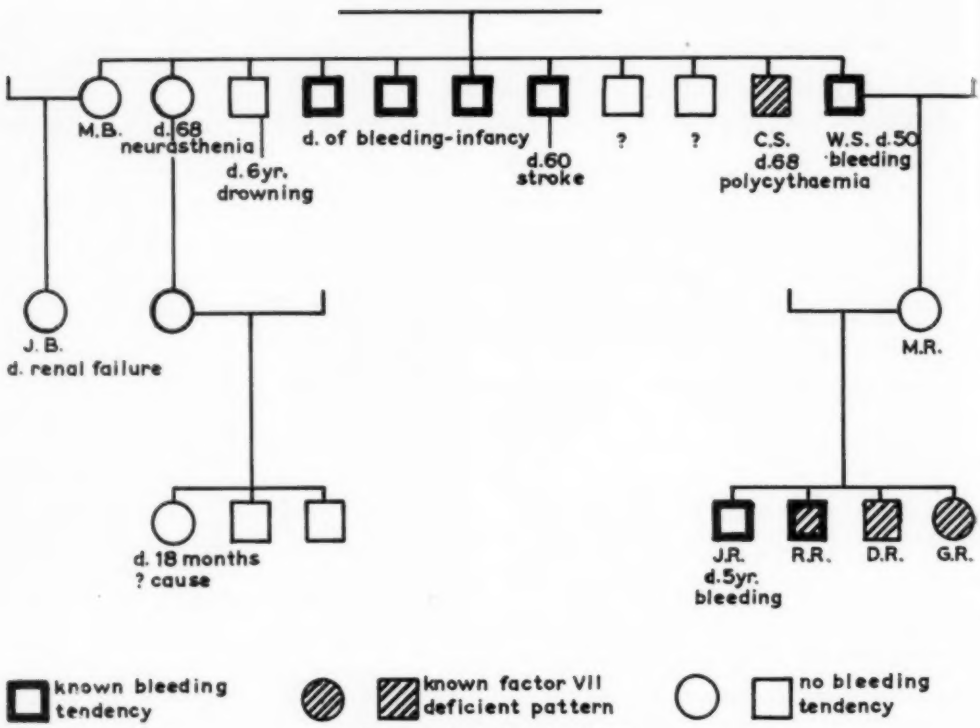

FIG. 1.—Pedigree of R and S families.

palpable at this time, but the facies suggested polycythaemia vera. Blood examinations supported the diagnosis of polycythaemia and showed: haemoglobin 121% (17.9 g. per 100 ml.), R.B.C. 8,330,000 per c.mm., W.B.C. 22,400 per c.mm., P.C.V. 85, E.S.R. 1 mm. in the first hour (Westergren), platelets 643,000 per c.mm. Liver function tests were normal. The Wassermann reaction and P.P.R. were negative. Hess's test was negative.

A sudden rectal haemorrhage led to his treatment in hospital for three weeks, the haemoglobin being 93% (13.8 g. per 100 ml.) and the P.C.V. 56 on discharge. He was then given radioactive phosphorus and remained well for three months, when he had a transient episode of auricular fibrillation. In September, 1958, he developed bronchitis and became disorientated, dying soon afterwards of a cerebrovascular accident.

Inquiry into the family history had shown that three brothers died of bleeding in infancy, while another brother suffered from bleeding episodes throughout life. Yet another brother, W.S., also a "bleeder," had severe haemarthroses and died aged 50 of internal bleeding. His daughter M.R. has had four children.

The R Family

M.R. shows no bleeding tendency and is in good health. John R. died aged 5 years of intra-abdominal bleeding following a fall. He had bled frequently since birth and severe haemarthroses had necessitated knee calipers.

Derek R. (aged 4) and Gillian R. (aged 18 months) are alive and well; neither have shown any bleeding symptoms or joint disorders.

Raymond R. is aged 9 years and has had several severe episodes of bleeding necessitating six blood transfusions. Bleeding has followed minor injury or dental extraction. He has moderately severe knee haemarthroses.

Laboratory Investigations

Material and Methods.—Blood was collected by clean venipuncture into one-ninth of its volume of 3.8% trisodium citrate and the plasma separated without delay by centrifugation for 20 to 30 minutes. Serum was obtained from blood allowed to clot in plain glass containers and incubated at 37° C. for two to four hours. Siliconed syringes and vessels were used in the collection of many of the plasma

specimens. Tests were carried out using standard laboratory procedures as described by Biggs and Macfarlane (1957). Prothrombin times were carried out by Quick's (1935) method using acetone-dehydrated brain thromboplastin. Triplicate readings were taken and the results expressed as to the nearest half-second. Blood fibrinogen was measured by the biuret method following precipitation by thrombin (normal range 200 to 400 mg.%).

Investigations were possible on M.B. and C.S. of the S family, and M.R., R.R., G.R., and D.R. of the R family. The relevant findings are shown in Tables I and II. M.R. and M.B. show no abnormality of blood coagulation. The bleeding times are within normal limits in all members. R.R. has shown an abnormal clotting time on several occasions. In C.S., D.R., G.R., and R.R. the one-stage prothrombin time is slightly prolonged. The change is "corrected" by the addition of normal stored serum. The qualitative tests for factor-V deficiency and the two-stage prothrombin time (Biggs and Macfarlane, 1957) show no evidence of deficiency in factor V or in the activity of prothrombin. The sera were less effective than normal stored sera in correcting the prolonged prothrombin times of plasma from patients receiving the anticoagulant drug phenindione ("dindevan" plasma). Serum from a case of Christmas disease* was as effective as normal serum in correcting the prolonged prothrombin times. The change appeared to be due to an alteration in the "factor-VII activity" of the sera.

Thromboplastin generation is abnormal in R.R. The results indicate that the defect is present in his serum, that admixture of his plasma with the plasma of a patient with true haemophilia produced normal thromboplastin generation, and that tests using the patient's serum and serum from a case of Christmas disease showed no mutual correction of the defects. It was concluded that the serious clotting abnormality in R.R. is Christmas disease, and that an associated change in "factor-VII" activity is also present in this boy, his brother and sister, and in Charles S.

TABLE I

RESULTS OF ROUTINE COAGULATION STUDIES OF THE FAMILY

	C.S.	M.R.	D.R.	G.R.	R.R.
Clotting time (min.) (Lee and White) ..	9½	13	9	6	50
Bleeding time (Ivy)	Normal	Normal	Normal	Normal	Normal
Platelet count (per c.mm.)	643,000	200,000	317,000	333,000	258,000
Fibrinogen (mg. %)	360	340	300	320	360
Prothrombin time (sec.)					
Patient plasma ..	15	12	14	14	16
Control ,, ..	12	12	12	12	13
Patient + 10% normal serum	11·5	—	12	12	13
Patient + 10% Christmas disease serum ..	12	—	12	12	13
Two-stage prothrombin time ..	Normal	Normal	Normal	Normal	Normal
Thromboplastin generation ..	Normal	Normal	Normal	Normal	Abnormal

TABLE II

THROMBOPLASTIN GENERATION IN PATIENT R.R. (USING NORMAL PLATELETS)

Incubation time (min.):	1	2	3	4	5	6
Incubation Mixture	Prothrombin Time (sec.)					
Normal plasma + serum ..	38	22	15	12	11	11
Patient's plasma + Normal serum ..	32	16	12	11	10	10
Patient's serum + Normal plasma ..	39	26	24	20	19	20
Patient's serum + Christmas serum + Normal plasma ..	36	27	24	23	22	21
Haemophilic plasma + patient's plasma + Normal serum	50	41	25	13	10	8

Further Investigations

In view of the finding of a slightly prolonged prothrombin time in Charles S., it was decided to study unrelated patients with polycythaemia vera. The prothrombin times, tests of liver function, and fibrinogen estimations were carried out in 10 patients with polycythaemia vera, two patients with erythroleukaemia (P.M. and E.H.), and one patient with chronic marrow failure following polycythaemia (E.S.). The prothrombin times were measured without making any special allowance for variations in the concentration of anticoagulant in their plasma. Many of the patients were under treatment at the time of testing and their packed cell volumes were not markedly increased. The results are shown in Table III. Abnormalities of liver function were noted in seven patients. The zinc turbidity tests were within normal range in all subjects and are not included in the table; the thymol turbidity or flocculation tests, however, were abnormal in several patients. Blood fibrinogen estimations in 12 patients did not show any significantly reduced values.

The one-stage prothrombin times were slightly but definitely prolonged in several patients, and in J.W., J.S., E.H., P.M., and E.S. the blood changes showed the features of "factor-VII" deficiency, as far as was indicated by the addition of 10% normal serum and by the effect of the serum or plasma on the prolonged prothrombin time of

*The serum was kindly supplied by Dr. Rosemary Biggs.

TABLE III

LABORATORY FINDINGS IN 13 CASES OF POLYCYTHAEMIA VERA AND ALLIED DISORDERS

Patient	Sex and Age	P.C.V.	Prothrombin Time to Nearest Second		Liver Function Tests				Fibrinogen (mg. %)
			Patient's Plasma	Control Plasma	Bilirubin (mg. %)	Alkaline Phosphatase (K.-A. units)	Thymol Turbidity (units)	Thymol Flocculation	
F.W.	M 57	32	16	13	0·5	8·3	3	Negative	220
C.H.	M 60	52	16	12	0·7	36·4	3	Negative	360
E.M.	F 79	39	15	13	0·7	11·8	1	Negative	250
C.K.	M 46	36	18	13	0·7	27·2	2	Positive	230
I.S.	F 60	44	13	13	0·5	8·3	1	Negative	210
F.T.	M 58	66	14	12	1·1	20·6	2	Negative	200
J.S.	F 54	61	13	13	0·6	7·2	6	Positive	270
J.S.K.	M 65	51	21	14	1·1	5·8	3	Negative	—
P.M.	F 47	48	15	13	<0·5	10·8	4	Positive	220
E.S.	F 75	51	17	13	0·6	10·8	1	Negative	370
J.P.	M 52	74	16	13	<0·5	12	5	Negative	400
E.H.	F 78	53	16	13	<0·5	9·5	5	Positive	430
J.W.	F 75	53	16	12	<0·5	9·5	3	Negative	250

"dindevan" plasma. Aluminium hydroxide-adsorbed normal plasma was ineffective in "correcting" the prothrombin times and the two-stage prothrombin times were normal.

The one-stage prothrombin time in E.S. was 17 seconds and in P.M. 15 seconds (control times 13 seconds) before and 24 hours after giving intramuscular vitamin K_1.

Mixtures of sera and plasma were tested to see whether any mutual correction occurred; such mixing procedures were carried out between the various members of the R and S families, between members of the family and unrelated polycythaemics, and between the polycythaemics themselves. In some cases, the prothrombin times for mixtures of plasma were measured directly; aluminium hydroxide-adsorbed normal plasma was added to the incubating mixtures as a source of factor V. In other tests, use was made of the ability of sera or plasmas or of mixtures to shorten the prolonged prothrombin times of "dindevan" plasma. Representative findings are shown in Tables IV and V. It was found that normal serum shortened the prothrombin times of "dindevan" plasma to a greater extent than sera and plasma of the various subjects or of mixtures of sera. Tests using mixtures of plasmas showed no significant mutual correction of the changes.

TABLE IV

PROTHROMBIN TIMES OF TEST PLASMAS AND MIXTURES OF TEST PLASMAS

		Prothrombin Time (sec.)						
		Source of Plasma (2 vol.)						
Normal 19		C.S. 22·5	R.R. 22	D.R. 23	J.P. 26	E.H. 27	J.W. 23	E.S. 23
		Source of Plasma (1 vol.)						
Source of plasma (1 vol.)		C.S.	D.R.	J.P.	E.H.	J.W.	E.S.
	R.R.	22	24	25	27	24	23
	C.S.	—	24	26	23	22	23·5
	D.R.	—	—	22	21·5	23	23
	Normal	19	19	19	19	19	19

The incubated mixture consisted of 2 vol. of test plasma or mixture of equal volumes of test plasmas, and 1 vol. of aluminium-hydroxide-adsorbed normal plasma.

TABLE V

EFFECT OF TEST SERA AND MIXTURES OF TEST SERA ON PROTHROMBIN TIMES OF DINDEVAN PLASMAS (TENTH DAY OF TREATMENT)

		Prothrombin Time (sec.)					
		Source of Serum or Saline (2 vol.)					
Dindevan plasma A (18 vol.)		Normal Saline 36	Normal Serum 20	C.S. 23·5	R.R. 28	G.R. 29	D.R. 23
Dindevan plasma B (18 vol.)		Normal Saline 44	Normal Serum 20	J.P. 24·5	J.W. 31		E.H. 23·5
		Source of Serum (1 vol.)					
Dindevan plasma A (18 vol.)	Source of Serum (1 vol.)		C.S.	D.R.	G.R.		
			R.R.	26·5	23·5	29	
			G.R.	25	23	—	
			D.R.	22·5	—	—	
Dindevan plasma B (18 vol.)			J.P.	J.W.	E.H.		
			C.S.	24·5	26	23·5	
			R.R.	25·5	31	25·5	
			D.R.	23·5	22·5	22	
			J.W.	23	—	25	
			E.H.	25	—	—	

Discussion

Although polycythaemia vera is occasionally familial (Nadler and Cohn, 1939) and Christmas disease shows classical haemophilia-like sex-linked inheritance, the occurrence of these blood dyscrasias within a family is probably coincidental. In reviewing familial leukaemia and other serious blood disorders, Guasch (1954) notes only occasional examples of the familial association of haemorrhagic states with other serious blood diseases. Instances of different blood disorders

occurring within one family are generally too few for any specific associations to be apparent.

The slightly prolonged prothrombin times in the present family may be an inherited change in the factor-VII activity of the blood. The changes have been noted in the R family in tests carried out on several separate occasions. The unaffected members of the families reported by Bell and Alton (1955) and Constandoulakis (1958) show similar variation in the one-stage prothrombin time. Although combined coagulation deficiencies are uncommon, a change in factor-VII activity has been most often noted in association with Christmas disease, itself of much lower incidence than classical haemophilia. The association may prove to be more frequent than can be accounted for by the chance association of separate inherited coagulation disorders. A further feature is that the changes in prothrombin time in these familial examples have been slight, and near the limits of normal variability. Ingram and Armitage (1959) have investigated the normal range of Quick's prothrombin time determinations and found the differences in prothrombin times of normal persons to be greater than can be accounted for by the experimental errors of the method. The causes of this variability and their possible relationship to minor changes of the factor-VII deficiency type are not yet fully explained.

The serum factors concerned in blood coagulation are complex, and their classification and interrelationships are uncertain. Apart from Christmas factor, several members with factor-VII-like activity have been distinguished; these include the Prower-Stuart factor (Telfer, Denson, and Wright, 1956), the factor deficient in cases of congenital factor-VII deficiency (Owren, 1952; Dische, 1958), and a further thromboplastin-forming substance, C (Biggs, 1956). These members of the complex have been intensively studied by using "mixing procedures": Bachmann, Duckert, Geiger, Baer, and Koller (1957) summarize the findings in 40 reported cases of factor-VII complex abnormalities; a number of cases remain unclassified and include those familial examples associated with Christmas disease. It is perhaps significant that these cases show only slight prolongations of the prothrombin time, contrasting with the grossly abnormal times of many of the other cases. Further work is necessary to clarify the relationships of these various members.

It is well recognized that minor prolongations of the prothrombin time, sometimes of the factor-VII deficiency pattern, are found in patients with malabsorption states or severe liver disease. The significance of the finding in the present cases of polycythaemia vera is uncertain. In the five patients investigated, the prolonged prothrombin time appeared to be due to a change in factor-VII activity.

Abnormalities of liver function tests were noted in some of the patients. The biochemical findings and the inability of vitamin K_1 to alter the prothrombin time in two of the patients suggests that the changes in prothrombin time may be an index of hepatic dysfunction recognized to be a not infrequent feature of polycythaemia vera (Tinney, Hall, and Giffin, 1943). As far as is indicated by the mixing procedures employed, no significant differences were noted between the changes in factor-VII activity of the blood of the members of the family studied and those observed in the patients with polycythaemia vera, or related disorders.

I am deeply grateful to Dr. T. P. Lalonde and Dr. R. H. Walker for their help and encouragement in investigating their patients of the R and S families; to Miss G. Penney for technical help, and to Mr. F. Grimes for the biochemical estimations.

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EFFECTS OF POSTURE ON PLASMA VOLUME AND SOME BLOOD CONSTITUENTS

BY

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When the posture was changed from horizontal to vertical, or the reverse, the alteration in plasma volume and in the levels of haematocrit, haemoglobin, and plasma protein was much greater in patients with oedema or low plasma protein or albumin concentrations (" the pathological group ") than in patients without these abnormalities (" the control group "). This larger fluid shift, and the larger concentration changes dependent on it, are explained in terms of Starling's hypothesis.

In the control group there was scarcely any change in the total mass of intravascular plasma protein with change in posture, but in the pathological group this was often considerable. This was interpreted as indicating increased permeability of the capillaries to protein.

Attention is drawn to the influence of uncontrolled alterations in posture on biochemical and haematological measurements and a recommendation is made in order to standardize conditions before taking blood for the analysis of non-diffusible constituents.

It has been known for many years that posture has a great effect on the concentration of certain blood constituents (Böhme, 1911) and on the plasma volume (Thompson, Thompson, and Dailey, 1928), but little account has been taken of such effects in clinical work. The concentration of plasma protein and red cells alters because, on changing from the supine to the erect position, fluid leaves the circulation under the influence of hydrostatic forces and the lower limbs swell (Waterfield, 1931b). In healthy people the plasma volume decreases by about 12% on changing from lying to standing (Thompson *et al.*, 1928; Waterfield, 1931a; Youmans, Wells, Donley, Miller, and Frank, 1934). This fluid shift is reversed on reverting to the horizontal position.

We became aware of the importance of these changes when investigating the variation during the course of the day of the blood levels of plasma protein and certain other substances (Fawcett and Wynn, 1956b). Early evidence suggested that these effects of posture might be greater in patients with oedema or abnormal plasma protein or albumin concentrations, and it was therefore decided to study the influence of posture in such patients. A control group of subjects, lacking these abnormalities but under similar conditions of admission to hospital, was used for comparison. The effects on the levels of plasma protein, haematocrit, haemoglobin, mean corpuscular haemoglobin concentration, total erythrocyte volume, and plasma volume are reported and their significance discussed.

Subjects and Analytical Methods

The patients (hereafter designated the pathological group) with oedema or abnormal plasma protein or albumin concentrations are described in Table I. The control group, maintained under similar hospital conditions, comprised 10 patients awaiting minor surgical operations or convalescing from them.

Blood samples were taken from a vein of the antecubital fossa into heparinized syringes, using 0.04 ml. of heparin (5,000 units per ml.) per 10 ml. of blood. When frequent sampling was required, a Guest's cannula was inserted into the vein so as to avoid repeated venepuncture and the necessity for venous occlusion. In separate venepunctures, minimum occlusion was used to insert the needle in the vein and the tourniquet was then released for about a minute before withdrawing blood.

Plasma protein was determined by a semi-micro Kjeldahl method (Fawcett, 1954). Albumin and globulin were estimated after precipitation of the latter with 26% sodium sulphate (Majoor, 1942, 1946; Fawcett and Wynn, 1956a). Haematocrit measurements were carried out in triplicate in capped Wintrobe tubes which were centrifuged at 3,000 r.p.m. for 55 minutes in a centrifuge of 15 cm. radius, correcting for trapped plasma according to the data

TABLE I
PATHOLOGICAL SUBJECTS

Patient	Diagnosis	Experiment	Oedema*	Proteinuria (g./day)	Plasma Protein† (g./100 ml.)	Albumin/ Globulin	Plasma Non-protein Nitrogen (mg./100 ml.)
A	Acute glomerulonephritis (Type I) ..		−	1	6·3	0·6	26
B	,, ,, ,, ..		−	26	5·1	0·3	32
C	Chronic glomerulonephritis with acute exacerbation		++	6	6·3	0·6	72
D	Chronic glomerulonephritis with acute exacerbation		++	2	6·8	0·5	62
E	Nephrotic syndrome (Type II nephritis) ..	i	++	1	4·4	0·6	36
		ii	++	5	4·0	0·9	45
		iii	++	4	4·6	0·8	40
F	Chronic hepatitis with portal hypertension	i	−	−	6·15	1·4	21
		ii	−	−	5·45	1·0	19
G	,, ,, ,, ,, ,,	i	−	−	8·95	0·4	22
		ii	−	−	8·05	0·7	20
H	,, ,, ,, ,, ,,	i	++	−	6·2	0·2	30
		ii	+	−	7·2	1·4	20
I	Chronic nutritional oedema	i	+	−	5·3	0·8	36
		ii	−	−	4·85	1·3	32
		iii	+	−	3·5	1·1	29
J	Idiopathic oedema		+	−	6·5	1·2	29

* Pitting of both legs up to the thighs, ++; pitting of both legs up to the knees, +. Only case H had ascites.
† Plasma total protein concentration at start of test (horizontal posture).
Sitting, instead of vertical, posture was used in experiments B and Hii.

of Chaplin and Mollison (1952). Blood haemoglobin was determined as oxyhaemoglobin (Bell, Chambers, and Waddell, 1945).

Total circulating erythrocyte volume was measured by labelling some of the subject's own blood cells with 20 μc. of $Na_2{}^{51}CrO_4$ (Gray and Sterling, 1950; Mollison and Veall, 1955) and these were reinjected 30 minutes before taking the first blood sample. The erythrocyte volume was then calculated from the concentration of labelled cells in this sample.

Plasma volume was measured with Evans blue dye, using the acetone extraction method of Chinard and Eder (1948), or with human plasma albumin labelled with ^{131}I. Ten minutes were allowed for equilibration of each tracer material and then at least four samples of plasma were obtained at intervals, the last being obtained between 30 and 60 minutes after the injection. The theoretical concentration at the time of injection was obtained by extrapolation, and the volume of distribution of the tracer was calculated.

Results

Effects of Posture on Plasma Protein and Haematocrit Levels.—In the standard experimental procedure each subject remained in bed for 12 hours and lay flat for at least one hour. Blood was taken and the subject then assumed the vertical posture for one hour. During this time the subject was slowly walking about, thus ensuring adequate circulation in the lower extremities but avoiding effects due to strenuous exertion. (Youmans *et al.*, 1934, observed that in stationary subjects haemoconcentration in the foot was greater than that in the arm.) Blood was taken 15 minutes and 60 minutes after rising. The subject then resumed the horizontal position, further samples being taken after 15 and 60 minutes. This procedure was used for 10 control subjects and for 12 experiments on nine pathological subjects. Less complete data were obtained in five additional experiments on pathological subjects, and in two of these the patients had to sit in a chair instead of adopting the usual vertical posture.

The percentage changes in protein and haematocrit levels are given in Table II. Fifteen minutes after rising from bed the plasma protein concentration increased by about 10% in both the control and pathological groups. After an hour there was scarcely any further rise in the controls, but in the pathological subjects the protein level rose higher, averaging 15% and in several patients about 20% above the resting values. There was no further increase in plasma protein concentration after one hour. The average decrease 15 minutes after lying down again was about 6% for the controls and about 9% for the pathological subjects. After an hour the changes were about 9% and 13% respectively. The percentage decreases after returning to bed were less than the corresponding increases because the protein concentration started at higher levels, but the volumes of fluid shift represented were the same.

Similar observations apply to the haematocrit changes, although these were smaller because they reflected alterations in blood volume instead of plasma volume. Fifteen minutes after rising, the haematocrit increased by 7 to 8% in both groups. After an hour, although there was no further rise

TABLE II

CHANGES OF PLASMA PROTEIN AND HAEMATOCRIT LEVELS AS PERCENTAGE OF VALUE BEFORE CHANGE OF POSTURE

Subjects	Experiment	% Change in Protein				% Change in Haematocrit			
		Vertical		Horizontal		Vertical		Horizontal	
		15 min.	60 min.	15 min.	60 min.	15 min.	60 min.	15 min.	60 min.
Control 1 ..		—	+5·0	−4·9	−10·5	—	+2·6	−3·4	−6·9
2 ..		+7·8	+9·3	−2·0	−3·4	+5·7	+4·8	−2·1	−3·6
3 ..		+11·4	+7·6	−1·4	−5·7	+6·1	+4·5	−3·0	−7·0
4 ..		+6·6	+7·6	−8·2	−9·7	+8·4	+8·6	−4·8	−6·3
5 ..		+6·0	+10·5	−9·5	−12·9	+3·6	+3·8	−5·5	−7·5
6 ..		+19·0	+13·9	−7·1	−11·5	+11·9	+5·4	−1·9	−5·8
7 ..		+10·2	+11·8	−7·7	−7·1	+7·9	+9·0	−7·2	−6·0
8 ..		+11·7	+13·9	−8·9	−9·7	+7·1	+7·0	−6·9	−7·2
9 ..		+7·6	+14·2	−5·3	−10·0	+6·2	+9·8	−3·1	−9·3
10 ..		+10·7	+10·7	−8·4	−11·0	+9·8	+10·0	−7·5	−9·9
Controls: Mean		+10·1	+10·5	−6·3	−9·2	+7·4	+6·6	−4·5	−7·0
S.D.		3·9	3·0	3·0	2·9	2·4	2·9	2·2	1·7
Patient A ..		+5·6	+10·3	−10·8	−13·0	+4·7	+6·9	−8·2	−10·5
B ..		—	+12·7	—	−12·3	—	+10·7	—	−9·7
C ..		+10·3	+15·9	−10·3	−13·0	+14·8	+18·1	−13·1	−12·6
D ..		+9·6	+12·5	−7·2	−13·1	+7·2	+15·0	−8·4	−13·5
E ..	i	+8·0	+12·5	−5·1	−11·1	+9·7	+11·5	−4·4	−9·9
	ii	+8·9	+15·0	−7·6	−19·6	+11·8	+12·5	−10·1	−14·4
	iii	—	+18·5	—		—	+10·7	—	—
F ..	i	+14·6	+19·8	−7·7	−14·5	+9·2	+14·1	−8·3	−12·3
	ii	+12·3	+18·0	−9·2	−17·1	+10·2	+17·1	−12·1	−17·4
G ..	i	+4·5	+10·6	−6·6	−11·6	+7·7	+12·5	−13·7	−14·9
	ii	+11·5	+9·9	−13·0	−12·4	+9·5	+8·8	−9·4	−9·4
H ..	i	+5·6	+11·3	−2·9	−8·0	+3·6	+9·4	−2·1	−8·5
	ii	—	—	—	−8·1	—	—	—	−8·6
I ..	i	+11·3	+13·2	−6·7	−12·5	+6·1	+7·1	−7·8	−14·1
	ii	+8·1	+17·8	−7·5	−17·0	+5·4	+12·3	−8·4	−16·2
	iii	—	+28·6	—	—	—	+16·7	—	—
J ..		—	+20·1	—	−16·5	—	+12·2	—	−12·0
Pathological group Mean* ..		+9·2	+15·4	−8·6	−13·3	+8·3	+12·3	−8·8	−12·3
S.D.* ..		3·0	5·0	2·8	3·2	3·2	3·4	3·3	2·7
Significance of difference between groups		0·1<p	p<0·02	0·1<p	p<0·01	0·1<p	p<0·01	p<0·01	p<0·01

* Excluding experiments B and Hii, in which sitting replaced the vertical posture.

in the controls, in the pathological subjects the haematocrit rose higher, averaging 12% and in several patients about 17% above the resting values. As with the protein changes, there was no further rise after one hour. The average decrease 15 minutes after lying down again was about 5% for the controls and 9% for the pathological subjects. After an hour the changes were about 7% and 12% respectively.

Effects of Posture on Mean Corpuscular Haemoglobin Concentration.—We have measured haematocrit in preference to haemoglobin because we found this can be done with greater precision. In terms of percentage changes the two move in parallel unless there is an alteration in the mean corpuscular volume which would be reflected in a change in the mean corpuscular haemoglobin concentration. To investigate whether this occurs, studies were carried out on haemoglobin and M.C.H.C. levels in five of the experiments described above, the subjects comprising four controls and one patient with the nephrotic syndrome. The results in Table III show that the mean level of M.C.H.C. was slightly (0.9%) greater in the horizontal posture than in the vertical, but the difference was not statistically significant.

Effects of Posture on Total Circulating Erythrocyte Volume.—A radioactive isotope technique was used to investigate the possibility that liberation and subsequent sequestration of

TABLE III

EFFECT OF POSTURE UPON MEAN CORPUSCULAR HAEMOGLOBIN CONCENTRATION (G./100 ML.)

Subject	Experiment	Posture		Percentage Difference
		Horizontal	Vertical	
Control 1		32·6	32·3	+0·9
,, 2		31·1	30·3	+2·6
,, 3		32·1	32·1	0
,, 4		32·3	32·1	+0·6
Patient E	(i)	33·2	33·0	+0·6
Mean				+0·9
S.D.				1·0
Significance of difference from zero				0·1<p

erythrocytes might contribute to the changes in haematocrit. In four experiments (Ei, Fii, Gi, and Ii) on the pathological subjects, in which the rise in haematocrit was an average of 12%, labelled erythrocytes were injected 30 minutes before the start of the experiment. It was found that the activity per unit volume of red cells fell by an average of 0.9% during the hour after rising. This represented a negligible fraction of the rise in haematocrit and was not significantly different from zero. There was thus no evidence to suggest that sequestered (and therefore unlabelled) erythrocytes were liberated into the circulation on assuming the vertical posture.

It was not possible to investigate in the same way the possibility of erythrocytes being removed from the circulation on lying down, because such sequestered cells would contain the same concentration of radioactivity as those remaining. It would be difficult, however, to postulate that such sequestration occurs on adopting the horizontal posture without the reverse process occurring on standing up.

Effects of Posture on Plasma Volume.—It is difficult to make direct measurements of changes in plasma volume because the methods are not very precise, and when repeated determinations are required in a short period the measurements are complicated and precision further reduced. This is equally true of both the dye and isotope methods. As both tracers are bound to plasma protein, and therefore influenced by its movement from the circulation and changes in its concentration, one cannot dispense with the necessity for two separate injections of the labelling material in order to calculate the response to a change in posture. The second measurement is therefore complicated by the rate of removal of the material first injected. One is thus attempting to assess a relatively small difference between two measurements neither of which can be made with very great precision.

An alternative method of calculating percentage changes in plasma volume (V) can be based on the changes in haematocrit, on the assumption that the total volume (E) of circulating erythrocytes and the ratio of total circulation haematocrit to venous haematocrit are unaffected by posture. This is because

$$V = E \cdot \frac{100 - c.Ht}{c.Ht} \quad \ldots\ldots\ldots\ldots (1)$$

where haematocrit (Ht) is expressed as a percentage, and c is a factor correcting for plasma trapped in the erythrocyte column of the haematocrit tube (Chaplin and Mollison, 1952) and for the difference between total circulation and venous haematocrit (Chaplin, Mollison, and Vetter, 1953).

The validity of this method of calculation depends not only upon the assumption that E is unaffected by posture, which we have confirmed at least within 1% limits, but also that c is unaffected. Only a considerable redistribution of blood between the large and smaller vessels could have a significant effect on c, and this is unlikely to occur.

In an attempt to confirm the validity of using this equation, experiments were carried out on three control and four pathological subjects to measure their changes in plasma volume, after a change in posture, both by direct measurements and by means of equation (1). Six direct measurements were made using Evans blue dye, injecting 15 mg. for the first estimation and 30 mg. for the second. Human plasma albumin labelled with ^{131}I was injected simultaneously with the dye in one of these six subjects and was used instead of the dye in a seventh subject. In two experiments the patients were not well enough to adopt the usual vertical posture for the time necessary to ensure complete adjustment and equilibration of plasma volume (60 minutes) plus that necessary for obtaining samples after the injection (at least 30 minutes). In these patients, the "vertical" posture was represented by sitting in a chair. In all experiments allowance was made for the plasma removed in the blood samples.

Table IV compares the percentage changes in plasma volume obtained by these direct measure-

TABLE IV

COMPARISON OF CHANGES IN PLASMA VOLUME IN RESPONSE TO CHANGE IN POSTURE CALCULATED BY DIRECT MEASUREMENTS OF PLASMA VOLUME AND FROM EQUATION (1)

Subjects	Label	Change Calculated as Percentage of Plasma Volume in Vertical Posture		
		Direct Measurement (a)	From Equation (1) (b)	Difference a − b
Control 1	Evans blue	+0·4	+3·0	−2·6
	^{131}I	+1·5		−1·5
,, 2	Evans blue	+9·9	+9·3	+0·6
,, 3	,,	+5·7	+5·6	+0·1
Patient E	^{131}I ,,	+16·9	+16·2	+0·7
,, H		+14·2	+15·4	−1·2
,, I	Evans blue	+33·6	+28·4	+5·2
,, J	,,	+17·0	+22·5	−5·5
Mean				−0·5
S.D.				3·1
Significance of difference from zero				0·1<p

^{131}I = ^{131}I-labelled albumin.

TABLE V

CHANGES OF PLASMA VOLUME AS THE PERCENTAGE VALUE BEFORE POSTURE CHANGE

Subjects	Experiment	Vertical		Horizontal	
		15 min.	60 min.	15 min.	60 min.
Control 1 ..		—	−5	+6	+12
2 ..		−10	−8	+4	+7
3 ..		−9	−7	+5	+12
4 ..		−9	−9	+9	+10
5 ..		−6	−7	+10	+15
6 ..		−17	−8	+3	+10
7 ..		−12	−13	+14	+11
8 ..		−11	−10	+12	+12
9 ..		−11	−16	+6	+19
10 ..		−12	−12	+11	+16
Controls: Mean		−10·8	−9·5	+8·0	+12·5
S.D.		3·0	3·3	3·2	2·8
Patient A ..		−7	−9	+13	+17
B ..		—	−16	—	+18
C ..		−15	−18	+17	+16
D ..		−9	−16	+11	+19
E ..	i	−13	−16	+7	+17
	ii	−17	−18	+19	+28
	iii	—	−14	—	—
F ..	i	−12	−18	+13	+21
	ii	−13	−20	+20	+29
G ..	i	−10	−15	+24	+25
	ii	−13	−13	+17	+17
H ..	i	−5	−12	+3	+12
	ii	—	—	—	+15
I ..	i	−8	−9	+12	+20
	ii	−7	−17	+15	+30
	iii	—	−23	—	—
J ..		—	−17	—	+22
Pathological group Mean* ..		−10·7	−15·7	+14·2	+21·0
S.D.* ..		3·4	3·6	5·0	5·4
Significance of difference between groups ..		0·1<p	p<0·01	p<0·01	p<0·01

* Excluding experiments B and Hii, in which sitting replaced the vertical posture.

ments and by means of equation (1). There is no evidence of a systematic difference and agreement is generally good in view of the inherent lack of precision of the direct measurements. We regard this as confirmation of the validity of using the equation to assess percentage changes in plasma volume. Changes calculated in this way from the haematocrit data in Table II are reported in Table V.

In the control group the average change on moving from the horizontal to the vertical posture was about −10% and the change on resuming the horizontal was about +12%. In most of the pathological group the plasma volume changes were greater, sometimes reaching 30%.

As with the plasma protein and haematocrit levels, the percentage changes in plasma volume on rising differed from those on returning to bed. This is because of the differences in the starting levels, not because of differences in the volumes of fluid transferred.

Effects of Posture on Total Mass of Intravascular Plasma Protein.—From our values for the percentage changes of plasma volume and protein concentration it is possible to estimate the corresponding changes in the total quantity (P) of plasma protein within the vascular circulation. This is because

$$P = V \cdot [Pr] \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

where [Pr] represents plasma protein concentration and from equations (1) and (2),

$$\frac{P}{E} = [Pr]\ \frac{100 - c.Ht}{c.Ht} \quad \ldots\ldots\ldots\ldots (3)$$

If E is constant, percentage changes in P are equal to percentage changes in the right-hand side of the equation.

Table VI shows the mean alterations in this P/E ratio. In our control group the alterations tended in the same direction as plasma volume but were very small. Therefore we believe that the total mass of intravascular plasma protein in these subjects was scarcely affected by posture,

TABLE VI

CHANGES OF P/E* AS PERCENTAGE OF VALUE BEFORE POSTURE CHANGE

Subjects	Experiment	Vertical		Horizontal	
		15 min.	60 min.	15 min.	60 min.
Control 1 ..		—	0	+1	+1
2 ..		−3	0	+2	+4
3 ..		0	−1	+4	+6
4 ..		0	0	−1	0
5 ..		0	+3	0	0
6 ..		−1	+5	−4	−3
7 ..		−3	−3	+4	+3
8 ..		+1	+3	+2	+1
9 ..		−4	−4	+1	+5
10 ..		−3	−3	+2	+3
Controls: Mean		−1·5	0	+1·1	+2·0
S.D.		1·8	2·6	2·5	2·8
Patient A ..		−2	+1	+1	+2
B ..		—	−5	—	+4
C ..		−6	−4	+5	+1
D ..		−1	−5	+3	+4
E ..	i	−7	−6	+3	+5
	ii	−9	−6	+11	+4
	iii	—	+2	—	—
F ..	i	+2	−2	+5	+4
	ii	−2	−6	+10	+8
G ..	i	−6	−7	+16	+12
	ii	−4	−4	+2	+3
H ..	i	0	−2	0	+2
	ii	—	—	—	+6
I ..	i	+2	+3	+5	+6
	ii	0	−2	+7	+10
	iii	—	−2	—	—
J ..		—	−1	—	+3
Pathological group Mean† ..		−2·7	−2·9	+5·7	+5·0
S.D.† ..		3·5	3·0	4·6	3·1
Significance of difference between groups ..		0·1<p	p<0·05	p<0·05	p<0·05

* P = total mass of intravascular plasma protein. E = total volume of circulating erythrocytes.

† Excluding experiments B and Hii, in which sitting replaced the vertical posture.

TABLE VII

ESTIMATED CHANGES OF ONCOTIC PRESSURE IN TERMS OF G. ALBUMIN PER 100 ML.

Subjects	Experiment	Vertical		Horizontal	
		15 min.	60 min.	15 min.	60 min.
Control 1 ..		—	+0·28	−0·28	−0·61
2 ..		+0·40	+0·47	−0·11	−0·18
3 ..		+0·54	+0·36	−0·07	−0·28
4 ..		+0·32	+0·36	−0·43	−0·50
5 ..		+0·29	+0·51	−0·51	−0·69
6 ..		+0·94	+0·69	−0·40	−0·64
7 ..		+0·47	+0·54	−0·40	−0·36
8 ..		+0·52	+0·64	−0·46	−0·49
9 ..		+0·35	+0·68	−0·28	−0·53
10 ..		+0·52	+0·52	−0·47	−0·60
Controls: Mean		+0·48	+0·50	−0·34	−0·49
S.D.		0·19	0·14	0·16	0·16
Patient A ..		+0·25	+0·46	−0·52	−0·63
B ..		—	+0·35	—	−0·38
C ..		+0·44	+0·67	−0·55	−0·65
D ..		+0·40	+0·50	−0·35	−0·60
E ..	i	+0·22	+0·35	−0·16	−0·35
	ii	+0·24	+0·42	−0·24	−0·63
	iii	—	+0·56	—	—
F ..	i	+0·68	+0·92	−0·43	−0·85
	ii	+0·45	+0·70	−0·42	−0·75
G ..	i	+0·24	+0·57	−0·40	−0·65
	ii	+0·63	+0·54	−0·75	−0·72
H ..	i	+0·18	+0·37	−0·10	−0·28
	ii	—	—	—	−0·47
I ..	i	+0·41	+0·48	−0·27	−0·51
	ii	+0·29	+0·62	−0·35	−0·70
	iii	—	+0·71	—	—
J ..		—	+0·94	—	−0·84
Pathological group: Mean ..		+0·37	+0·57	−0·38	−0·60
S.D. ..		0·16	0·18	0·18	0·17
Significance of difference between groups ..		0·1<p	0·1<p	0·1<p	0·1<p

possibly varying by about 1% of the total intravascular pool. In many of the pathological cases, the P/E ratio varied much more than in the controls, decreasing in the vertical posture and increasing in the horizontal position. In four of the experiments the change in the P/E ratio was between 10 and 16% of the initial value, representing some 20 to 30 g. of plasma protein. It can be seen from Table VI that the fall in the P/E ratio in the pathological group averaged 2.9% 60 minutes after rising. On return to the horizontal position P/E rose an average of 5.0% in 60 minutes.

Effects of Posture on Plasma Oncotic Pressure.—We did not attempt to measure plasma oncotic pressure directly, but, for the limited purpose of comparing the changes in our two groups of subjects, we considered it justifiable to calculate on two assumptions: (*a*) that the oncotic pressure of plasma was entirely dependent on protein concentration, and (*b*) that globulin, as estimated by our salt-fractionation method, exerted two-fifths of the osmotic effect of an equal mass of albumin. No attempt was made to convert the values into units of pressure: they were expressed as changes in protein concentration, correcting globulin to its albumin equivalent. Thus if an observed change in protein concentration was 1.1 g. per 100 ml. and the A/G ratio was 6/5, then the change in plasma oncotic pressure would be represented as $0.6+(2/5\times0.5)=0.8$ g. of albumin per 100 ml. Table VII shows that the changes in oncotic pressure, calculated in this way, were similar in the pathological subjects to those in the control series.

Discussion

These results may be assessed in the light of both their physiological significance and their application to the clinical interpretation of laboratory reports.

The quantitative interpretation of our data, describing the movement of fluid and plasma protein to and from the blood, depends upon the assumption that change of posture is not accompanied by a significant alteration in the volume occupied by the erythrocytes in the circulation or a large alteration in the total circulation: venous haematocrit ratio, or alternatively that any alterations which may occur are similar in health and in disease. We tested this assumption by repeated measurements of M.C.H.C., of the total circulating erythrocyte volume, and of the plasma volume, and we concluded that the haematocrit may be used to calculate changes in plasma volume under the conditions of these experiments, even in disease, and even when the changes were very large. Systematic errors in such calculations were unlikely to exceed about 1% of the plasma volume.

The changes in plasma protein concentration could not always be used in the same way as the haematocrit because, although large, they were sometimes less than were to be expected from the haematocrit changes. We interpreted this as indicating movement of protein to and from the circulation. For example, in the six instances in which the P/E ratio altered within 15 minutes by between 7 and 16%, this would represent the movement of some 15 to 30 g. of plasma protein. The capillary wall is the most probable site at which this quantity of plasma protein could be transferred to and from the circulation in such a short period, and we suggest that increased permeability of the capillaries is therefore indicated in these patients.

According to Starling's hypothesis, the distribution of fluid between capillary blood and the interstitial fluid depends predominantly on the

hydrostatic pressure within the capillaries being balanced by the plasma oncotic pressure. Our observations are consistent with this because, although the movements of fluid in our pathological cases were larger than in our controls, the changes in plasma oncotic pressure were very similar. In patients with plasma protein abnormalities or increased permeability of the capillaries to protein, larger volume changes are required to bring about the same changes in plasma oncotic pressure, thus balancing the postural changes in hydrostatic pressure. This explains the larger fluid shifts in such subjects.

Because of the large changes in plasma volume which may quickly occur, it is obvious that posture affects the haematocrit, haemoglobin, and other haematological levels, and the concentration of plasma protein, cholesterol, and other relatively non-diffusible constituents, including calcium. As emphasized by Widdowson and McCance (1951), it is important for these effects to be taken into account in clinical work. For example, we have known patients with hypoproteinaemia in whom the diagnosis was not made because they had been walking about before blood was taken for analysis, and in whom the plasma protein levels were much higher than they were subsequently found to be when the patients were confined to bed. Similarly, serial observations on haemoglobin or serum calcium may be invalidated if uncontrolled alterations of posture are allowed to influence the results.

We have found that intermediate changes in posture have less effect on the composition of blood than the horizontal-vertical alterations reported in this paper. It is therefore suggested that, in order to standardize the influence of posture, patients should be sitting up in bed or in a chair for at least a quarter of an hour before blood is withdrawn for haematological, plasma protein, or other investigations which might be affected by changes in plasma volume.

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MULTIPLE CONGENITAL COAGULATION DEFICIENCIES

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A 6-week-old infant is presented who suffered from a congenital haemorrhagic disorder which caused death from subdural haemorrhage following mild trauma. Haematological investigation revealed deficiencies of factor VII and Christmas factor. Prower-Stuart factor was probably also deficient although investigation of this clotting factor was carried out only on serum obtained at necropsy.

Multiple congenital deficiencies of coagulation factors occur infrequently and only a small number of such cases have been reported in the literature (Constandoulakis, 1958). The combined deficiencies of factor VII and Christmas factor have been described by several authors (Bell and Alton, 1955; Stein and Abrahams, 1956; de Vries, Kettenborg, and van der Pol, 1955; and Nour-Eldin and Wilkinson, 1959), but none of these investigators has carried out tests to exclude the possibility of an additional deficiency of Prower-Stuart factor. The infant reported in this paper is of interest because all three of these factors are believed to have been deficient. Factor X has not been considered because Denson (1958) has produced evidence to suggest that it is identical with Prower-Stuart factor. The case is also of some clinical interest as an example of the occasional important relation between traumatic intracranial haemorrhage and coagulation defect. It has already been briefly reported in that connexion (Simpson and Robson, 1960).

Clinical Summary

A.T.U., aged 6 weeks, a boy, was admitted to the Adelaide Children's Hospital on July 29, 1959. The parents stated that he had been mildly unwell for several days. On the day of admission he had vomited and passed several loose stools. The right face and arm had twitched. He had become progressively more drowsy, and by the time he came into hospital he barely responded to painful stimuli. On particular inquiry, the parents recalled that the baby had received a trivial bump to the head on the previous day. On several occasions they had noticed that the baby developed bruises with undue ease; he had also bled excessively from scratches and from a small infection on the foot.

On examination, he was found to be deeply unconscious. The fontanelle was stony hard. There was an obvious paralysis of the left third nerve; the left pupil was dilated and unreactive, the right pupil reacted sluggishly. The pulse was slow (70–80) and the respiration periodic. A diagnosis of acute subdural haematoma was made, and immediate operation advised.

While preparations were being made, it was noted that the blood clotting time was prolonged. This confirmed the suspicion of a coagulation disorder. The parents, who were intelligent people, knew of no other bleeders among their near relatives; their other child was wholly normal in this respect.

Operation was carried out under general anaesthesia. Burr holes were made in the left frontal and parietal regions. Fluid and solid blood were found in the subdural space under great pressure; it was evacuated without difficulty, but the exact volume could not be measured. A right frontal burr hole disclosed no abnormality. At this stage the child's general state worsened, and it was decided that osteoplastic decompression would not be tolerated.

During operation, fresh frozen plasma (130 ml.) and blood (about 30 ml.) were infused, and later more blood was given to a total of perhaps 250 ml. When the baby left the theatre, the conscious state had improved, but soon after this he became markedly shocked, and, despite transfusion and the use of vasopressor drugs, he died several hours later. The pre-operative haemoglobin was estimated at 7.0 g.%. The platelet count, total leucocyte count, and examination of a stained blood smear were all within normal limits.

Necropsy

The examination was carried out eight hours after death. The body was that of a male infant weighing 4,750 g. with extreme pallor of the skin and mucous membranes. There were four or five bruises situated on both knees, the sternum, and the right side of the scalp.

Three surgical incisions were present on the scalp and blood oozed from these wounds although they were firmly sutured. A scratch mark with a well-formed scab was present on the right cheek and there was a drip incision over the left medial malleolus. The right great toe nail had a little clotted blood under one margin. The diaphragm was at the level of the sixth rib on the left and the fifth rib on the right side. The liver weighed 166 g., was soft, mottled, brownish-red, and on incision was yellowish-brown and moist. The spleen was purple, weighed 15 g., and the Malpighian bodies were visible. The heart was pale and the chambers contained an unusually small amount of blood. Burr holes were present on either side of the frontal bone and another was situated over the left parietal bone. A subdural blood clot approximately 5 cm. × 3 cm. × 0.3 cm. was situated over the left cerebral hemisphere. Underlying this was a subarachnoid film of blood. On incision an area of softening, approximately 3 cm. in diameter, was seen in the parietal lobe extending from the posterior central gyrus to the angular gyrus. In the right cerebral hemisphere there was a thin subarachnoidal film of blood covering the parietal lobe. A localized widening of the central sulcus was evident and on incision two small haemorrhagic areas about 5 mm. in diameter were present in the putamen. The leptomeninges covering the vermis and left lobe of the cerebellum were discoloured a dark orange. Pallor of the trachea was marked. No other naked-eye abnormality was seen.

Histological examination of the liver showed an occasional very early micro-abscess with an excess of leucocytes in the sinusoids. Leucocytes were excessive in the splenic sinusoids. The bone marrow showed megakaryocytic hyperplasia. In the brain, subarachnoid and intracerebral haemorrhages were evident, with necrosis and softening of the adjacent brain tissue.

Investigation of the Coagulation Abnormality

Materials and Methods.—A small amount of blood was obtained from a cannula passed directly into an arm vein which was exposed before transfusion. Approximately 1 ml. was placed in a sterile bottle and allowed to clot at room temperature. Serum was separated after storage overnight at 37° C. The remainder of the blood obtained was mixed with one-ninth volume of 3.8% sodium citrate solution and the plasma was separated by centrifugation at 3,000 r.p.m. for 10 minutes.

Plasma deficient in factor VII was obtained from the patient previously reported in detail (Hicks, 1955) who suffered from a congenital deficiency of this factor. The plasma was stored at −20° C. before use. Serum from a patient with Christmas disease was stored similarly. Dindevan plasma was collected from a patient who had been treated with this drug for three weeks. $Al(OH)_3$-treated plasma was obtained by mixing fresh plasma with one-ninth volume of $Al(OH)_3$-gel and agitating at 37° C. for four minutes before separation by centrifugation.

The one-stage prothrombin test of Quick (1935) was carried out using a 5% suspension of acetone-treated human brain.

The "stypven" time was determined by substituting "stypven" (Burroughs Wellcome & Co.), to which lipoid had been added, for the brain extract in the one-stage prothrombin test.

The two-stage prothrombin test (Biggs and Douglas, 1953a) was modified as described by Hicks (1955), and in addition an equal volume of serum was added to supply adequate quantities of factor VII and Prower-Stuart factor.

The thromboplastin generation test and the thromboplastin generation screening test were carried out as described by Biggs and Douglas (1953b) and Hicks and Pitney (1957) respectively.

Results

Whole-blood Clotting Time.—Blood collected as described above took a little longer than one hour to coagulate. The clot which formed was firm, of normal consistency, and retracted well without any appreciable fibrinolysis.

Quick's One-stage Prothrombin Test. — The patient's plasma showed a markedly prolonged clotting time when tested by Quick's method. Mixing experiments were carried out as shown in Table I. The defect in the patient's plasma was almost corrected by the addition of 20% of normal plasma. It was improved by adding 50% of normal serum, but $Al(OH)_3$-treated plasma had no effect. The patient's serum did not improve the prolonged one-stage time of factor-VII-deficient plasma.

TABLE I

EFFECTS OF PLASMA AND SERUM MIXTURES ON ONE-STAGE PROTHROMBIN TIME

Normal Plasma (%)	Patient's Plasma (%)	Normal Serum (%)	$Al(OH)_3$-treated Normal Plasma (%)	Patient's Serum (%)	Factor VII-deficient Plasma (%)	One-stage Prothrombin Time (sec.)
100						11
	100					>600
20	80					15
	80	20				60
	50	50				22
	50		50			68
			100			63
					100	47
				20	80	46
				50	50	47

Thromboplastin Generation Studies.—A gross defect in the patient's plasma was demonstrated by the thromboplastin generation screening test. Mixing experiments were therefore carried out as shown in Table II. It can be seen that normal serum corrected the defect, but even when as much as 20% of undiluted $Al(OH)_3$-treated normal plasma was added to the incubation mixture, little or no improvement occurred. Factor-VII-deficient plasma also corrected the abnormality, but serum deficient

TABLE II

EFFECTS OF ADDED PLASMA OR SERUM ON THROMBOPLASTIN GENERATION SCREENING TEST

Normal Plasma Diluted 1/10 (%)	Patient's Plasma Diluted 1/10 (%)	Normal Serum Undiluted	Normal Serum Diluted 1/10 (%)	$Al(OH)_3$-treated Normal Plasma Undiluted (%)	Factor VII-deficient Plasma Diluted 1/10 (%)	Christmas-factor-deficient Serum Diluted 1/10 (%)	Screening Test: Minimum Clotting Time (sec.) of Six Successive Min. Subsamples
100							9½
	100						70
	80	20					10½
	80			20			35
Tests carried out after storage at −20° C.							
100							10½
	100						40
	80		20				15
	50		50				12
	50				50		12½
	50					50	28

in Christmas factor had little beneficial effect. Similar results were also obtained with the thromboplastin generation test of Biggs and Douglas (1953b). However, insufficient plasma was available for treatment with $Al(OH)_3$-gel and experiments were therefore carried out on the patient's serum only, in the presence of normal $Al(OH)_3$-treated plasma and lipoid platelet substitute. The defect did not appear as pronounced with this test as in the thromboplastin generation screening test. A minimum substrate clotting time of 16 seconds was obtained when the patient's serum was examined.

Two-stage Prothrombin Test.—Prothrombin was found to be present in adequate quantities. The areas enclosed by the curves in Fig. 1 are proportional to the amounts of prothrombin in the patient's plasma and in plasma from a normal subject.

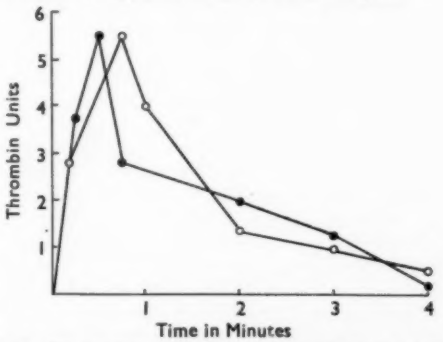

FIG. 1.—The two-stage prothrombin test was performed on normal plasma (●——●) and a plasma from the patient (O——O). An equal volume of serum was added to supply adequate quantities of factor VII and Prower-Stuart factor.

Stypven Clotting Time.—At this stage of the investigation no plasma or serum taken from the patient before death remained and these tests were carried out using serum obtained from the right ventricle at necropsy eight hours after death. The results are tabulated in Table III, which shows that the addition of the patient's serum did not appreciably shorten the "stypven" time of the "dindevan" plasma.

TABLE III

EFFECT OF PATIENT'S SERUM ON VIPER VENOM TIME OF DINDEVAN PLASMA

Normal Plasma (%)	Dindevan Plasma (%)	Patient's Serum (%)	Russell's Viper Venom Time (sec.)
100			10½
	100		25½
	80	20	21½
	50	50	22

Discussion

The investigation of this patient was hampered by the very small amount of blood available for testing. Approximately 1 ml. of plasma and 0.5 ml. of serum were obtained. The number of tests carried out was therefore limited to those considered most essential for the determination of the disorders present.

The one-stage prothrombin test revealed a striking abnormality. The results of the mixing experiments shown in Table I indicate that normal serum contained a factor (or factors) capable of considerably improving the defect. Factor VII and Prower-Stuart factor are present in normal serum and are concerned in the reaction between plasma and tissue thromboplastin. Therefore, a deficiency of either or both of these factors could have been responsible for the patient's prolonged one-stage clotting time. A deficiency of factor VII was shown conclusively when the patient's serum and a plasma sample known to be deficient in factor VII only were not mutually corrective. The viper venom time is prolonged with plasma deficient in Prower-Stuart factor but remains normal when factor VII is absent.

Dindevan plasma was used as a source of plasma deficient in Prower-Stuart factor, and the fact that this and the patient's serum were not mutually corrective for the viper venom time (Table III) suggests that the patient was deficient also in Prower-Stuart factor. It was unfortunate that there was insufficient patient's plasma to estimate the viper venom time directly, and that the serum used had to be obtained after death. It is difficult to be certain of the reliability of work carried out on serum obtained after death, although three other sera similarly obtained at necropsy contained adequate amounts of Prower-Stuart factor. This suggests that the absence of this factor from the patient's serum was not due to post-mortem deterioration, and transfusion during life had not raised the levels to normal values.

Normal plasma was found to have a more pronounced effect than normal serum in correcting the prolonged one-stage time in Table I. It was felt that this indicated an additional deficiency of a factor normally present in plasma but not in serum. However, the two-stage prothrombin test (Fig. 1) revealed normal amounts of prothrombin, and mixing experiments involving $Al(OH)_3$-treated normal plasma (Tables I and II) failed to demonstrate any deficiency of factor V. We can therefore offer no satisfactory explanation of this phenomenon. The pattern of results was not consistent with the presence of an anticoagulant substance nor with any appreciable degree of fibrinogen deficiency.

Thromboplastin generation studies provided evidence of a further clotting factor deficiency. The results shown in Table II indicate that normal serum contained a factor (or factors) which corrected the patient's defective thromboplastin generation and which was not present in normal plasma after absorption with $Al(OH)_3$-gel. This finding could therefore have been due to the probable deficiency of Prower-Stuart factor or to an additional deficiency of Christmas factor. A deficiency of the latter was demonstrated by the failure of serum from a case of Christmas disease to correct the defect. It was noted that factor-VII-deficient plasma corrected the thromboplastic abnormality in the patient's plasma, but this was to be expected because factor VII is not concerned in thromboplastin generation.

A similar but less severe disorder is sometimes associated with liver disease, but the changes found in the liver of this patient were not sufficient to cause a serious dysfunction. The age of the infant was inconsistent with a diagnosis of haemorrhagic disease of the newborn.

We wish to thank Dr. E. B. Sims and the board of management of the Adelaide Children's Hospital for permission to publish this case report.

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THE BLOOD GROUPS OF GASTRIC ULCER-CANCER

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There was a statistically significant excess of Group A blood in a series of ulcer-cancers. It is concluded that an ulcer in a patient of Group A is more liable to become malignant than one in a patient of Group O.

It has been shown by a number of authors that there is an excess of blood Group A in carcinoma of the stomach and Group O in chronic gastric ulcer. Consequently, it has been postulated that Group A antigen possesses a carcinogenic effect and Group O a protective effect against cancer. If this is true then malignant change in a chronic gastric ulcer should occur more often in patients of Group A than of Group O. If not, then the group distribution in ulcer-cancer would be the same as in chronic gastric ulcer from which it originates.

However, whether or not malignancy ever develops in a chronic gastric ulcer has been debated for over a century. Some authors consider that the change never occurs and others that most cancers arise from chronic ulcers. This difference of opinion depends largely on the criteria adopted to decide whether or not there is evidence of a pre-existing gastric ulcer in the carcinoma.

The wide criteria of MacCarty and Broders (1914) led them to believe that 472 out of 684 cancers of the stomach possessed the characteristics of simple ulcer with carcinoma. Dible (1925) presented much more definite histological evidence of the existence of previous ulceration in a gastric cancer. From a study of chronic gastric ulcers, he listed three characteristics: (1) Muscular destruction; (2) endarteritis or organizing thrombi; (3) extensive fibrosis. He qualified these to some extent by saying that carcinoma can give rise to endarteritis and that fibrous tissue may be due to a scirrhous carcinoma. The presence of a continuous band of muscle, in Dible's opinion, excluded a pre-existing ulcer. In 33 gastric carcinomata he found no evidence of preceding ulcer in 28, and in the remaining five he was not certain histologically whether or not the ulcer had preceded the cancer, and he felt that the appearance might have been produced by the sclerosing character of the growth. Turning to clinical evidence, he noted that a long history had been obtained in only two cases (five years and 20 years respectively), and he thought it hardly admissible to adhere to a histological diagnosis in the face of contrary clinical evidence.

In the same year (1925), Cabot and Adie had decided, as the result of long experience and of a survey of the literature, that a history of previous ulceration was unreliable for judging the relationship between ulcer and cancer. They also quote Kocher, who claimed that he had personally examined the Mayo Clinic specimens and was convinced that much of what Wilson and MacCarty (1909) labelled as carcinomatous degeneration was atypical proliferation of epithelium or only epithelial changes in the progress of the ulcer and had nothing to do with carcinoma. In their assessment of ulcer-cancer, Cabot and Adie used criteria given them by W. S. Warthin, and these were induration or cicatricial fibrosis extending through the stomach wall, sclerosis of the blood vessels, and a scar, the greater part of which was free from cancer cells.

Newcomb in 1925 stated that in chronic ulcers the muscularis mucosae and muscularis lose their normal parallel relationship and, because of cicatricial contractions, fuse or approximate near the edge of the ulcer. He thought that this was proof of the previous existence of an ulcer.

A great deal of work, over many years, on this subject of the development of cancer in chronic gastric ulcer was carried out by Stewart. In 1926 he published his findings and agreed with the criteria laid down by Dible (1925). He thought that the complete destruction of the muscle coat of the greatest importance and stated that relics of the muscle coat persist in all early cancers and in many fairly advanced ones, but they are not

seen in chronic gastric ulcer. In only very rare cases do secondary ulceration and sloughing of a carcinoma extend through a tumour and destroy all traces of the original muscularis. He added that dense cancer-cell-free fibrous tissue is not produced by carcinoma, and, whilst complete destruction of the fibrous floor by cancer is possible, it would more likely be seen only in very advanced malignant disease. He pointed out that cancer usually begins in a chronic ulcer in the edge and then spreads outwards from and around the ulcer, rather than into the floor. In several of his specimens he thought there was evidence that the malignant change had originated in the floor rather than in the edge of the ulcer. End-arteritis he considered as of little value in the absence of the other signs of chronic gastric ulcer, though it was less common in primary gastric carcinoma. He noted the termination of the frayed ends of the muscularis in the walls of the ulcer, or at a later stage turning up into the floor near the margin, but did not describe it as approximation or fusion of the muscularis mucosae and muscularis.

In 1930, Newcomb again laid great stress on the histological sign of fusion and added that it was "the only certain criterion for the diagnosis of ulcer cancer," and that "it was suggested that fusion of the muscularis mucosae with the muscularis is as important a point in the diagnosis of ulcer-cancer as the finding of spirochaetes in syphilis."

Stewart (1931), in a further report, reiterated his criteria and again emphasized the presence of areas of fibrosis free from cancer cells. He stated that "no conclusive evidence is available, on the other hand, that peptic ulceration of a primary carcinoma is capable of producing a zone of cancer-cell-free fibrosis in the floor." In discussing the relationship of the muscularis mucosae and muscularis, he thought that if fusion were insisted upon a number of true cases of ulcer-cancer would be described as primary carcinoma. It was his opinion that fusion was evidence of healing and that carcinoma could undoubtedly develop in ulcers where there was no indication of healing.

In 1932, Newcomb gave an extensive review of the vast literature on this subject, demonstrating the wide diversity of views on the development of cancer in a chronic gastric ulcer. To establish evidence of chronic ulceration, he examined 161 chronic gastric ulcers, though he included five in which the muscularis had not been completely destroyed. His criteria were, largely, those of Dible (1925) and Stewart (1926), but he stressed the great importance of fusion or approximation of the muscularis mucosae and muscularis, which he said was first described by Turnbull, adding that he found it in 159 of the 161 ulcers (98.8%). Nevertheless, he mentioned that Stewart had observed in uncomplicated gastric ulcers that the two muscle layers were widely separated. He summarized his opinion of fusion or approximation by the statement that "this criterion is as valuable as the demonstration of tubercle bacilli in the diagnosis of tuberculosis."

Gömöri (1933) carried out a histological investigation of carcinoma arising in gastric ulcers. His criteria agreed with those of Dible, Newcomb, and Stewart, except that in his control series of chronic gastric ulcer, unlike Newcomb, he found fusion or approximation of the muscularis mucosae and muscular layer not in almost all ulcers but in 56 out of 64 (87%). He thought that the fusion or approximation depended largely on the phase which happened to prevail at the time of examination. In his report he quoted Borrman, who believed that it is rare for carcinoma to develop in an ulcer; but Borrman accepted only those lesions in which a small part of the ulcer base was occupied by tumour and in which the ulcer base was free from malignant cells.

Ewing (1936) believed that gastric digestion and infection could reduce the original cancer to a narrow rim of malignant tissue lying beneath the overhanging edge of an ulcer, and went on to state that islands of cancer cells in the edge of an ulcer were no indication that the cancer cells were preceded by the ulcer.

In 1938, Klein, who investigated a large mixed series of gastrectomy and post-mortem specimens, reported on eight that appeared macroscopically to be ulcer-cancers. He thought that three of them could be instances of the development of carcinoma in a chronic gastric ulcer. His Cases 4 and 5, he agreed, looked to be typical chronic peptic ulcer on one block, but further blocks showed infiltration of the base by carcinoma and this sign he regarded as against the diagnosis of ulcer-cancer. Of the remaining three, two he described as ulcerated cancers and one was a benign peptic ulcer. He completely disagreed with Newcomb that close approximation or fusion of muscle and muscularis mucosae was pathognomonic of pre-existing chronic ulcer, and believed that all signs of chronic peptic ulcer would be produced in a malignant tumour by the process of peptic digestion.

Doubt concerning the development of cancer in chronic gastric ulcers was expressed by Palmer (1939) in whose opinion fibrosis, destroyed muscle, fusion or approximation of muscularis mucosae and muscle layer, and endarteritis were not pathognomonic of a pre-existing benign ulcer, and "any or all of them may be found with primary carcinoma." His Fig. 2 is described as a "cross-section through ulcerating carcinoma showing scar tissue, floor, and general architecture of benign ulcer." This picture is a ×6 magnification and has the appearances of a chronic gastric ulcer, yet he states that it may be attributed to peptic digestion of carcinoma.

In a subsequent report with Humphreys, in 1944, he repeated his opinions, and these two authors stated that, as healing processes are so commonly seen in peptic ulceration, one should not be surprised to find similar healing in peptic ulceration occurring in tumour tissue. These illustrations and descriptions of Palmer and Humphreys (1944) were considered by Stewart (1953) to be by no means wholly convincing.

Mallory (1940) concluded that peptic ulceration of carcinoma *in situ* is usually interpreted as evidence of carcinoma arising in an ulcer.

In 1947, Stewart returned to the difficulty caused by superficial ulceration of a scirrhous cancer, but he pointed out that it did not resemble a chronic gastric ulcer and that in the scirrhous type the muscularis mucosae often persists. Some years later, in the Macewen Memorial Lecture of 1953, Stewart, in discussing this same diagnostic point, said that in an active ulcer, oedema, inflammatory cells, and fibrosis could so widen the submucosa that separation of the muscularis mucosae and muscularis would follow rather than approximation. On the same occasion he mentioned subserosal fibrosis with islets of fat, and stated that this "is not met with, in my experience, in primary carcinoma, however deeply ulcerated."

Fusion of the muscularis mucosae and muscularis was referred to by Morgan and Lee (1954), who considered it a valuable sign, but thought that its absence did not exclude the diagnosis of ulcer-cancer. They, too, noted that fusion could be destroyed by tumour.

Figures concerning the number of carcinomata that arise in gastric ulcers have varied widely since this subject was first discussed in the middle of the nineteenth century. In his article in 1932, Newcomb listed 102 reports on this subject published during the period 1845–1925. He quoted the opinion of Rokitansky (1842), who said that it sometimes occurred with simple ulcer and that it might arise from the ulcer; Zenker (1882) that most cases of gastric carcinoma arise in a pre-existing ulcer; Duplant (1898) that the grafting of carcinoma on an ulcer is impossible. In half of these 102 reports quoted by Newcomb, the authors claimed that not more than 10% arose from gastric ulcers, while 15 gave a figure greater than 50%. However, in the opinion of the majority (74 reports) 20% of carcinomata showed evidence of previous gastric ulceration, and, from the numbers and percentages given, Newcomb was able to calculate that 17.5% was an average.

Table I shows the figures for ulcer-cancers quoted by some of the papers published since 1925.

TABLE I

PERCENTAGE OF ULCER-CANCERS IN PREVIOUS SERIES

	No. of Gastric Cancers	No. of Ulcer-cancers	% of Ulcer-cancers	Methods of Diagnosis
Dible (1925)	33	2	6	Histological and clinical
Stewart (1926)	97	17	17·5	Histological
Hurst and Stewart (1929)	70	11	15·7	,,
Stewart (1931)	109	17	15·6	,,
Newcomb (1932) ..	46	6	13	,,
Gömöri (1933) ..	26	6	23	,,
Walton (1936)	461	44	9·5	Histological and clinical
Finsterer (1939) ..	673	96	14·2	Histological
Stewart (1947)	281	51	18·0	Clinical
Grimes and Bell (1950) ..	171	24	14·0	,,
Swynnerton and Truelove (1951)* ..	375	24	6·5	Histological
Ekström (1952)	138	14	10·2	,,
Stewart (1953)	581	103	17·7	,,
Morgan and Lee (1954)	104	2	2	,,
Gilmour (1958) ..	168		13·7	,,

*Expressed less certainty about this in 1952.

Dible (1925) had invoked the history of dyspepsia to help in the diagnosis of ulcer-cancer and had excluded this diagnosis because of a short history in three patients. In their book *Gastric and Duodenal Ulcer* (1929) Hurst and Stewart showed that a chronic gastric ulcer could exist without causing serious symptoms, and that a short history did not necessarily preclude the possibility of ulcer. Newcomb (1932) felt that it was wrong to assume that a malignant change arose in a gastric ulcer merely because it had a long history.

Doubts concerning malignant change taking place in chronic gastric ulcers have been expressed. Zachary Cope (1925) said that "there seemed little evidence that a simple ulcer ever ended in carcinoma." There was no doubt in the mind of Schindler (1941), who did not believe that gastric ulcers ever became malignant, and added that as 3–4% of all adults die from carcinoma of the stomach it was to be expected that the same

FIG. 1.—Fusion of muscularis and muscularis mucosae in a chronic gastric ulcer (× 7½).

percentage of all ulcer patients would develop a carcinoma also. Palmer and Humphreys (1944) doubted if a gastric ulcer ever developed malignant change. In 1951 Swynnerton and Truelove wrote that they had found 24 ulcer-cancers histologically proved, but in the following year they expressed less certainty about them. Sir Heneage Ogilvie in 1952 said, "I myself have never seen an undoubtedly innocent peptic ulcer change to a cancer," and further, "I believe it is the cancer that caused the ulcer, rather than the ulcer that caused the cancer." His explanation was that they were scirrhous cancers which had been attacked by the gastric juice and then undergone healing in those parts where all the malignant cells had been digested away. However, he admitted that it was not possible to distinguish between simple and malignant ulcers radiologically and clinically.

I think it would be agreed that only a histological examination can show if an ulcer preceded a carcinoma and, even then, it is not always possible to be certain, so figures based on investigations other than histological are even more liable to error. Then again, there are many stomach cancers that are never resected because of the inoperability of the disease, and it is not unlikely that some of those might have originated in chronic gastric ulcers. At the same time, figures of malignant change in ulcers derived from the percentage of ulcer-cancers of all resected gastric ulcers are equally erroneous, for to be true the number of ulcer-cancers should be related to all gastric ulcers, many of which do not come to operation.

Methods

Ulcer-cancers.—This series of ulcer-cancers was obtained by examining the routine histological sections

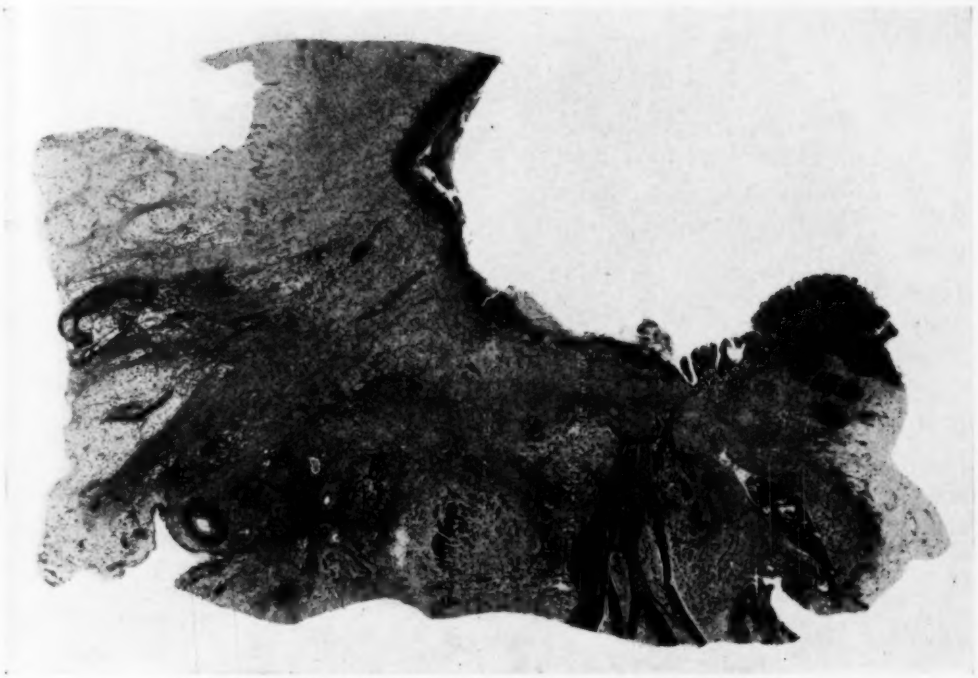

FIG. 2.—Approximation of muscularis and muscularis mucosae in an ulcer-cancer (× 7).

of 708 gastric cancers removed by gastrectomy or at necropsy. The diagnosis was made without knowledge of clinical facts or blood groups.

As has already been shown, many reports have been published that set out criteria for the diagnosis of ulcer-cancer. However, I decided to re-examine these criteria, in the light of information obtained from routine histological sections from 88 gastric ulcers removed surgically. All possessed the feature of a complete breach in the main muscle coat and this breach was filled by fibrous and granulation tissue.

The relationship of the muscularis mucosae to the main muscular coat was observed. The mingling of the muscle fibres of these was called fusion (Fig. 1). When the muscle fibres of both approached each other but did not fuse, this condition was called approximation (Fig. 2). Then there were some cases where the muscularis mucosae and muscularis still remained parallel, i.e., there was neither fusion nor approximation (Fig. 3).

Of the 88 ulcers in this series neither fusion nor approximation was found in 19, but fusion or approximation was observed in 69, although in several ulcers extension of the ulceration appeared to have obliterated evidence of fusion or approximation (Fig. 5). This gives a percentage of 78.4 compared with Newcomb's 98.8 and Gömöri's 87.

Chronic inflammatory changes often lead to the development of obliterative changes in the vessels in the floor of an ulcer. All reports that mention this feature point out, however, that it has little diagnostic value, but may have some usefulness as corroborative evidence (Fig. 6). More than a slight degree of endarteritis was found in 51 of these 88 ulcers (58%) and there was no endarteritis in 12 of them. Newcomb found it in 72% of his series. In primary carcinoma the comparable findings were that 12% of 649 had more than a slight degree of endarteritis and 73.1% had none. My results confirm its unreliability as a sign of previous chronic gastric ulceration.

One of the pitfalls in the diagnosis of ulcer-cancer is the misconstruing of irregularities in the size and shape of cells and nuclei and of mitoses in the glands in the edge of the ulcer as evidence of carcinoma. This may account for the fact that some authors have reported a high incidence of malignant changes in chronic gastric ulcer. For the purposes of this investigation, it was decided to accept only definite cases of cancer and in no instance was a diagnosis made on such cellular irregularities.

Difficulties also arose when there was superficial ulceration of a scirrhous carcinoma, and, in contrast, when masses of tumour cells obliterated the normal anatomy, and made it impossible to find any smooth

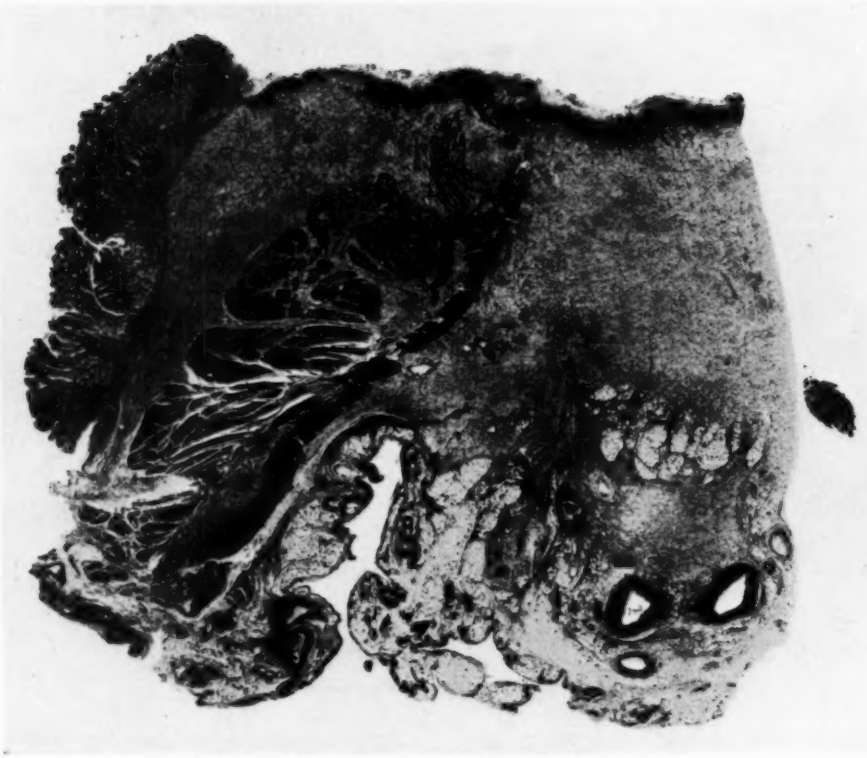

FIG. 3.—Neither fusion nor approximation in an ulcer-cancer (× 7½).

muscle. Then, however, the absence of areas of cancer-cell-free fibrosis and scar tissue placed the tumour in the primary group.

Scar tissue containing no cancer cells has been emphasized as a diagnostic feature. Spilsbury (1922) argued that cancer cells were unlikely to invade sclerotic and poorly vascular tissue, but are much more likely to spread centrifugally into the softer and highly vascular tissues around the ulcer. I think this is rather too rigid a criterion, for the scar tissue in the floor of chronic gastric ulcers shows much variation in vascularity. Some show many vessels and others a few, and some have a more cellular fibrous tissue than others. Newcomb (1932) observed these differences. Thus it would be logical to expect that cancer cells could invade the floor of an ulcer along the course of blood vessels in those cases where the floor is vascular, and, if the invasion were great enough, occupy a large area of the floor of the ulcer. In several sections it was noticeable that cancer cells were numerous near the peritoneal surface and extensions upwards into the scar tissue could be observed along the courses of vessels and nerves.

The criteria I looked for to indicate previous chronic gastric ulceration in a carcinoma were: (1) A complete breach in the muscle coat; (2) the occupation of this breach by fibrous and granulation tissue; (3) fusion and approximation of the muscularis mucosae and muscularis; (4) areas of cancer-cell-free fibrosis.

Chronic Gastric Ulcers.—The case records from a number of hospitals were examined and from these 690 cases of chronic gastric ulcer, which had been diagnosed histologically, were obtained together with their blood groups.

Carcinoma of the Stomach.—I was able to find from the clinical records of a number of hospitals the blood groups of most of the cases of carcinoma of the stomach that I examined in order to find ulcer-cancers.

Results

In a total of 708 carcinomata of the stomach 59 ulcer-cancers were found. The blood groups were available in 57.

FIG. 4.—Destruction of fusion or approximation by tumour in an ulcer-cancer (× 7).

The essential criterion was that there should be a gap in the muscle coat and this gap occupied by scar tissue. This was observed in all cases.

Cancer-cell-free fibrosis was noted in all, except that in four it was not as marked as in the others.

Fusion or approximation was observed in 32 ulcers and in 18 ulcers neither feature was observed. In four it appeared that neoplastic tissue had destroyed fusion or approximation (Fig. 4) and in one that it had been destroyed by ulceration.

In four cases the only sections available were of the floor of the ulcer and did not show the edge of the ulcer.

Endarteritis of more than a slight degree was seen in 40 cases, a slight degree in 13, and none in six.

Though this investigation was not planned to find the percentage of cancers that are derived from ulcers, the overall percentage of ulcer-cancer compared with the total gastric cancer is 8.3%, which is less than the figures quoted by Stewart (1926; 1931; 1947; 1955), Newcomb (1932), Gömöri (1933), and Walton (1936), but is a little higher than that of Dible (1925) whose figures were considered by Willis (1953) to be nearer the truth.

It is clear that there is an excess of Group A in the ulcer-cancer, for there are proportionately almost as many Group A bloods in ulcer-cancer as there are Group O in chronic gastric ulcer (Table II).

TABLE II

TABLE OF BLOOD GROUPS OF ULCER-CANCER AND OF CHRONIC GASTRIC ULCER

	O	A	B	AB	Total
Ulcer-cancers ..	21 (36·8%)	29 (50·9%)	7 (12·3%)	—	57
Histologically proved gastric ulcers	372 (53·92%)	254 (36·81%)	49 (8·10%)	15 (2·17%)	690

FIG. 5.—Destruction of fusion or approximation by ulceration in a chronic gastric ulcer (× 7).

Of the ulcer-cancers 50.9% and of the gastric ulcers 36.8% were Group A, a significant difference of 14.1 ± 6.7.

Discussion

This work has shown that there is a significant excess of blood Group A amongst cases of ulcer-cancer when compared with cases of benign chronic gastric ulcers. Whether or not a chronic gastric ulcer can become malignant is strongly disputed, and many believe that all the features of a chronic gastric ulcer can be reproduced by a cancer: that the fibrosis, muscle gap, fusion and approximation of muscularis mucosae and muscularis could be attributed to peptic digestion of a malignant tumour. It is believed that the evidence presented in this series supports the view that malignant change occurs in a chronic gastric ulcer.

In four of this series of ulcer-cancers, there were histories of indigestion for 20 years, 20, 30, and 40 years respectively. If it is assumed that ulcer-cancers are cancers from the outset, then it follows that peptic digestion had been taking place for those long periods of time and that the cancer had remained apparently localized to the stomach for the greater part of that time. This is difficult to accept. In cancer, peptic digestion causes a superficial necrosis and inflammatory response and it appears unlikely that the destroyed tumour is replaced by scar tissue. Similarly the invasion and destruction of the muscularis by neoplastic tissue are not followed by the formation of fibrous and granulation tissue.

It has been suggested that scar tissue is impermeable to cancer cells and that therefore the presence of cancer cells in the scar tissue supports the diagnosis of primary cancer of the scirrhous type. The bases of chronic gastric ulcers vary widely in their cellularity and vascularity, and cancer with its known characteristic of spreading along perivascular lymphatics could extend into a scar along the course of blood vessels and, if the scar were very vascular, could produce extensive tumour infiltration in the base of an ulcer.

Short histories of a gastric disorder are not exceptional in chronic gastric ulcer, and most pathologists, at some time or another, must have found, unexpectedly, a chronic gastric ulcer at necropsy. Thus, too much emphasis ought not to be placed on a long duration of clinical symptoms in the histological diagnosis of an ulcer-cancer.

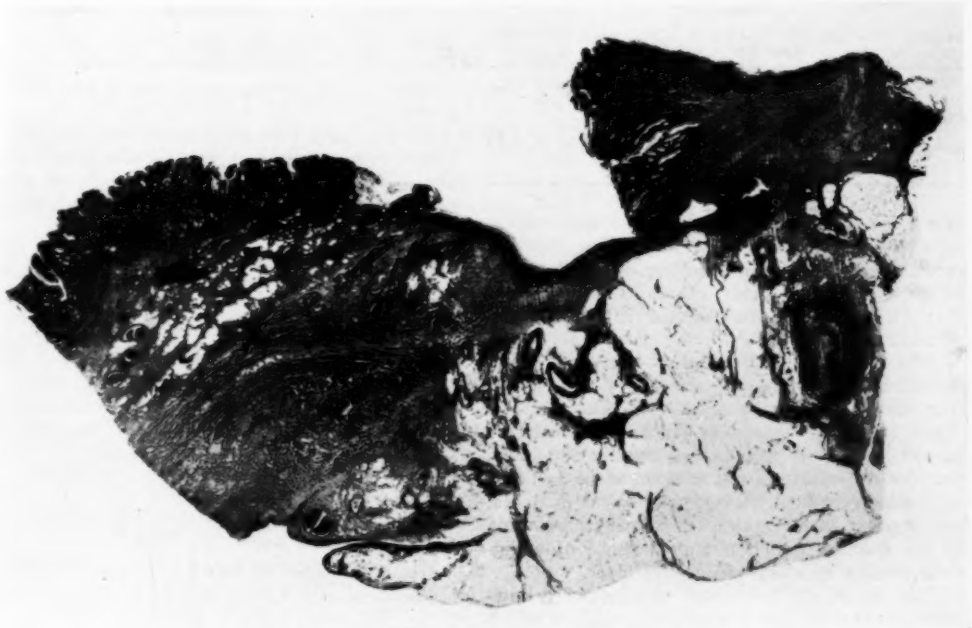

FIG. 6.—Endarteritis and fat islets in a chronic gastric ulcer (× 7½).

Therefore, if the histological appearances of the ulcer-cancers with the longest histories can be closely matched with those of others with shorter histories, then I have concluded that in all of them the lesion was originally the same, i.e., a chronic gastric ulcer.

In the margin of many chronic ulcers, and not only in those in the stomach, there is often some degree of irregularity of the epithelium. The cells show a variation in size and shape, stain more deeply and exhibit increased mitotic figures, and these features can give rise to difficulty in diagnosis. It is believed that cancer develops from these atypical foci, eventually spreading through the muscularis mucosa into the submucosa. In one case, invasion of the muscularis mucosa had only just begun, but an adjacent lymph node contained a small metastasis, thus making a convincing histological diagnosis. One patient, not included in this series, had had a partial gastrectomy for chronic gastric ulcer, but four years later he was found to have carcinoma of the gastric stump. Re-examination showed definite cellular irregularity at the edge of the ulcer but no invasion of the muscularis mucosae. This may be dismissed as a coincidence, but Helsingen and Hillestad (1956) pointed out that after partial gastrectomy for chronic gastric ulcer there was a risk of developing cancers in the gastric remnant three times greater than after partial gastrectomy for duodenal ulcer. They gave no pathological details, but knowing that duodenal ulcers seldom, if ever, become malignant it perhaps can be assumed that malignant changes must have occurred in the original gastric ulcer, but that they were not apparent to the surgeon or pathologist at the time.

It is concluded, therefore, that the histological evidence supports the belief that malignant change can occur in a chronic gastric ulcer and that ulcers in patients of blood Group A are more likely to develop cancer. This conclusion receives some support from animal experiments. Willis (1953) quotes the work of Deelman (1924), who found that wounds of a tarred area of skin localized and hastened tumour formation. Thus it would appear reasonable that, when in a stomach, already predisposed to malignant change by blood Group A, an ulcer develops, that ulcer becomes the precursor of a carcinoma.

There is, however, another possible interpretation of the excess of Group A in ulcer-cancer.

TABLE III

BLOOD GROUPS OF ULCER-CANCER AND PRIMARY GASTRIC CANCER

	O	A	B	AB	Total
Ulcer-cancer ..	21 (36·8%)	29 (50·9%)	7 (12·3%)	—	57
Primary cancer ..	228 (44·01%)	236 (45·56%)	38 (7·34%)	16 (3·09%)	518

When the ABO blood groups of the ulcer-cancer series are compared with those of primary gastric carcinoma (Table III) it can be seen that there are no great differences in the group distribution, and that Group A is predominant in both. The percentage of Group A in the ulcer-cancers was 50.9 and in primary cancer 45.6, giving an insignificant difference of 5.3 ± 7.0. This shows that there is statistically no difference between the ABO group in the ulcer-cancer and primary cancer groups. It perhaps can then be concluded that both diseases are one and the same and that ulcer-cancer is, in spite of histological evidence, just another manifestation of primary carcinoma of the stomach and is not an instance of malignant change in a chronic gastric ulcer.

This apparently reasonable conclusion is, however, contrary to the histological evidence which demonstrates quite convincingly that malignant changes do occur in a chronic gastric ulcer and therefore the possession of Group A blood rather than Group O predisposes that ulcer to the development of cancer.

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My special thanks are due to Dr. G. S. Andrews for his advice on the preparation of this paper.

Addendum

Since this paper was submitted for publication, I have obtained seven further examples of ulcer-cancer. Three belong to Group O and four to group A.

In the comparison of ulcer-cancers and chronic gastric ulcers there is now a significant difference of 14.75 ± 6.3, and in the comparison of ulcer-cancer and primary cancers there is an insignificant difference of 6.0 ± 6.6.

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A COMPARISON OF THE ROSE-WAALER, LATEX FIXATION, "RA-TEST," AND BENTONITE FLOCCULATION TESTS

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The bentonite flocculation test of Bozicevich, Bunim, Freund, and Ward (1958), the latex fixation test of Singer and Plotz (1956), and the "RA-test" (a latex reagent for use as a slide test) of Hyland Laboratories have been compared with each other and with a modified Rose-Waaler test, the behaviour of which has been previously extensively investigated. In these tests sera from 2,250 patients were tested by two or more methods on 3,000 occasions. The findings of this trial are set out and the merits of the tests and reasons for disagreement among them are discussed. It is concluded that the most satisfactory means of testing rheumatoid sera is by the Rose-Waaler test and the "RA-test," or a satisfactory modification of it, in parallel.

The presence in the serum of most patients suffering from rheumatoid arthritis of a substance capable of agglutinating red cells sensitized with homologous rabbit antibody, and of combining with Cohn fraction II (γ-globulin), is well established. This substance, now generally known as rheumatoid factor, is a high molecular weight globulin which in serum is found in combination with, and separable from, smaller γ-globulin molecules (Franklin, Holman, Müller-Eberhard, and Kunkel, 1957).

The rheumatoid factor occurs in a high proportion of rheumatoid sera and, with the exception of the "collagen diseases," in very few other conditions; consequently laboratory tests for its presence are becoming increasingly used as an aid to diagnosis and prognosis. Chief among the tests now in use are the sensitized sheep cell (Rose-Waaler) test in its many modifications; a variety of tests relying on the aggregation of latex particles by rheumatoid factor in the presence of fraction II, and the bentonite flocculation test (Bozicevich *et al.*, 1958) consisting of the agglutination of particles of bentonite pretreated with fraction II and dyed with methylene blue. There are many other methods of demonstrating the rheumatoid factor, for some of which great sensitivity has been claimed, notably tests using the euglobulin fraction rather than whole serum and the demonstration of an absence of an inhibitor of the Rose-Waaler test in rheumatoid sera; these tests, however, together with several others, are not widely used.

During the past six years we have conducted, with Dr. A. G. S. Hill, Director of the Oxford Regional Rheumatism Research Unit, and others, an investigation into the clinical significance of the Rose-Waaler test in a large series of patients, including 500 cases of rheumatoid arthritis. We have also performed many tests for other clinicians in this and other hospitals. With this experience as a basis it seemed helpful to compare other readily available tests in parallel with the Rose-Waaler test. The tests chosen for the comparison were the latex fixation test of Singer and Plotz (1956), the "RA-test," which is a commercially produced latex reagent for use as a slide test, and the bentonite flocculation test (Bozicevich *et al.*, 1958), since it seemed that these were the most suitable for routine laboratory use. The findings in a comparative series are presented here.

Clinical Material

Sera from 2,250 patients have been tested on more than 3,000 occasions. The patients from whom the sera were derived fall into two groups: those from the Rheumatism Research Centre (900) and those from all other sources (1,350). In the former group the diagnosis available has been the best possible, being arrived at after careful and repeated examinations and modified as necessary. The diagnosis used in compiling the data for the present survey has been that arrived at at the most recent assessment. In the latter group, the only diagnosis avail-

able has been that given on the laboratory request form received with the specimen.

Diagnostic Criteria

Patients from the Rheumatism Research Centre have been classified in respect of rheumatoid arthritis by criteria slightly modified from those of the American Rheumatism Association. It should be noted that a diagnosis of rheumatoid arthritis by these criteria does not imply that the case is either severe or long-standing, and indeed many cases in this series have minimal clinical signs and have been tested within a few weeks of the onset of the disease.

Laboratory Materials and Methods

Sheep Cell Agglutination (Rose-Waaler) Test.—The method was that described by Greenbury (1957). The test is done in perspex agglutination trays. After inactivation for 20 min. at 56° C. sera are diluted in saline containing 2.5% sheep serum in two-fold steps from 1 in 2 to 1 in 1,024. Two series of dilutions of each serum are made; to each dilution of one series is added an equal volume of 1% sheep cells sensitized with rabbit anti-sheep cell serum, and to the other (control) series a volume of 1% unsensitized cells. The trays are incubated at 37° C. for one hour and are then kept at 4° C. overnight. Readings are made next day by observing the pattern of sedimented cells on the bottom of the cups. The test is regarded as positive if the serum, absorbed if necessary, agglutinates sensitized cells at a dilution of 1 in 16; however, the great majority (96%) of positive sera have titres higher than 1 in 32.

The essential feature of this method is the application of the highest possible dose of rabbit anti-sheep cell serum for sensitizing the sheep cells. This considerably increases the sensitivity of the test without any loss of specificity. In the preliminary titration of the rabbit antiserum the dilutions are separated by a factor of only $\sqrt{2}$, in order to determine as accurately as possible the highest concentration of antiserum which can be used without causing agglutination of the red cells. Rabbit antiserum at this concentration is added to an equal volume of a 2% suspension of sheep cells, the resulting mixture being the 1% sensitized cells used in the test.

Latex Fixation Test.—The method used was that of Singer and Plotz (1956). To dilutions in buffer of the serum under test is added an equal volume of a buffered suspension of uniform latex particles, containing 0.04% fraction II (γ-globulin). After two hours at 56° C. the tubes are centrifuged and inspected for aggregation of the latex particles. In the present series, however, sera were tested only at a single dilution, 1 in 20. It was found that the best results were obtained if, after centrifuging, the deposits were resuspended and the tubes centrifuged a second time. This did not materially increase the number of unexpected positives and made reading considerably easier. Results range from a complete negative, the latex particles remaining evenly suspended and any precipitate being easily resuspended, to complete aggregation of the particles with a crystal-clear supernatant.

Bentonite Test.—This was a modification of the method described by Bunim and his colleagues (Bozicevich *et al.*, 1958) which in outline is as follows.

A suspension of bentonite particles of a suitable size range is obtained by differential centrifugation and to it is added 1% fraction II in buffer. After 15 min. the fraction-II-coated particles are washed and a small quantity of methylene blue is added. After rewashing, the now deep blue particles are suspended in phosphate buffer and a small quantity of Tween 80 is added. In the original method one drop of sensitized bentonite suspension was added to drops of a serial dilution of the serum to be tested on a large microscope slide. The slide was then placed on a rotating machine and inspected for agglutination after 20 min. In the present series only one dilution (1 in 20) of the serum was tested. The tests were done on the platforms (1 in. × 0.3 in.) of a grooved slide (Murray, 1944). The slides were placed in a tray which was rocked by hand for 20 min., or until agglutination of the particles in negative controls became imminent, whichever was the shorter period.

Hyland " RA-test."—This is a commercially produced latex/fraction II reagent for use as a slide test. The reagent is a milky suspension dyed pale green. The makers recommend that one drop each of the reagent and a 1 in 20 dilution in glycine buffer of the serum under test be mixed on a glass slide, which is then rocked back and forth. The results are read after 1 min. The results vary from no change to complete aggregation of the particles. Tests were done on grooved slides as described under the bentonite test. Any degree of unmistakable aggregation has been taken as positive. The best results were obtained if readings were made after 3 min. instead of 1 min. as recommended by the manufacturers, although strongly positive sera produce marked aggregation with complete clearing of the surrounding fluid within a few seconds.

Slide Latex Reagent (see Discussion).—This is made up as follows:

(1) *Polysterene Latex Particles.*—Diluted to form stock suspension (Singer and Plotz, 1956), this is about 1% solids.

(2) *Glycine Buffer.*—0.1 M Glycine in 1% NaCl adjusted to *p*H 8.2.

(3) 0.5% γ-Globulin in glycine buffer.

(4) Masson's light green.

Preparation.—To 3 ml. buffer add 0.2 ml. of 0.5% γ-globulin solution. Heat at 60° C. for 3 min. Cool and add 2 ml. latex particle suspension and 1 drop Masson's light green. This reagent has been used in the same way as " RA-test " reagent.

Results

All sera were submitted to the Rose-Waaler test and all but a few to the latex fixation test. On about the first 2,400 specimens the bentonite test also was used, after which, and for the rest of the series, the " RA-test " was included; after a further 300 specimens the bentonite test was dropped.

In all, sera from 2,250 patients were tested on 3,250 occasions with two or more tests. The majority of patients were tested only once, although many, especially those with rheumatoid arthritis, were tested on several separate occasions.

In order to provide a firm basis for the comparisons with the Rose-Waaler test which follow, a summary of our previous experience of the accuracy of our modification of this test is given in Table I. (These figures refer to a series investigated in great detail with Dr. A. G. S. Hill, Director of the Oxford Regional Rheumatism Research Centre, and other colleagues, to be published.)

TABLE I
RESULTS WITH MODIFIED ROSE-WAALER TEST

Diagnosis			Rose-Waaler Results +	Rose-Waaler Results −
Rheumatoid arthritis	Radiograph positive	Cases	336	33
		%	91	9
	Radiograph negative	Cases	101	30
		%	77·1	22·9
	All cases	Cases	437	63
		%	87·4	12·6
	First test	Cases	410	79
		%	83·8	16·2
Not rheumatoid arthritis		Cases	11	373
		%	2·8	97·1

In the first three rows in Table I a patient has been scored positive if he has been sero-positive on any single occasion during the period of observation. That is to say, about 10% of rheumatoid patients whose results in the Rose-Waaler test fluctuate from time to time have been scored positive. In order to keep the issue as clear as possible, cases of "possible" and "probable" rheumatoid arthritis, also psoriatic arthropathy, "collagen diseases," and generalized osteoarthritis, which may mask or be confused with rheumatoid arthritis, have been omitted from this table.

The results show this modification of the Rose-Waaler test to give a very high positive rate in rheumatoid arthritis and a low proportion of what may be false positives. It therefore forms a suitable base with which to compare the efficiency of other tests.

Table II compares the results obtained with all pairs of tests, including repeats on the same patient. In compiling this table tests giving equivocal or atypical results have been omitted. They are as follows: Rose-Waaler 25 (0.78% of all tests), tube latex 88 (2.6%), bentonite 50 (1.7%), "RA-test" 15 (1.8%).

In all pairings the agreement is in the region of 90%. This, however, is a slightly unflattering figure since there was a tendency to repeat tests more frequently on patients with whose sera the tests gave discordant results.

The results obtained with each of the tests related to a diagnosis of rheumatoid arthritis and to the source of the patient are given in Table III. These figures refer to a single test on each patient. In order to minimize any advantage there might be in favour of the Rose-Waaler test due to our longer experience with it, the results chosen for inclusion in Table III have been those of the latest test on each patient. Cases of "possible" and "probable" rheumatoid arthritis have been classified as "not rheumatoid arthritis": this accounts for the high incidence of positive results in this group.

The proportion of positive Rose-Waaler results in the rheumatoid arthritis group from the Rheumatism Research Unit agrees well with those recorded in Table I. (There is in fact some overlap in the material from which these tables are compiled.) Note the lower proportion of positive results in rheumatoid arthritis patients coming from sources other than the Rheumatism Research Unit.

Since the margin of disagreement between the tests is small a further comparison between pairs of tests is given in Table IV.

TABLE II
COMPARISON OF TESTS BY PAIRS

		(a)* + +	(b) − −	(c) + −	(d) − +	Agreements (a) + (b)	Total Tests
Rose-Waaler compared with latex test	Tests	1,156	1,737	185	144	2,893	3,222
	%	35·9	53·9	5·7	4·5	89·8	
Rose-Waaler compared with bentonite test	Tests	964	1,511	170	80	2,475	2,725
	%	35·4	55·5	6·2	2·9	90·9	
Rose-Waaler compared with "RA-test"	Tests	326	408	35	35	734	808
	%	40·4	50·6	4·3	4·7	91	
Latex compared with bentonite test	Tests	917	1,498	126	101	2,515	2,642
	%	34·7	56·7	4·8	3·8	91·4	
Latex compared with "RA-test"	Tests	333	392	44	30	725	799
	%	41·7	49·1	5·5	3·7	90·8	
Bentonite compared with "RA-test"	Tests	141	138	3	23	279	305
	%	46·2	45·3	1	7·5	91·5	

* The two signs in each column refer respectively, in order, to the results of each of the pairs of tests named in the first column.

TABLE III
TEST RESULTS RELATED TO DIAGNOSIS OF RHEUMATOID ARTHRITIS

Source	Diagnosis		Results of Tests							
			Rose-Waaler		Bentonite		Latex		"RA-test"	
			+	−	+	−	+	−	+	−
Rheumatism Research Unit	Rheumatoid arthritis	Cases	350	75	305	73	338	73	146	28
		%	82·3	17·7	80·7	19·3	82·2	17·8	83·9	16·1
	Not rheumatoid arthritis*	Cases	52	402	42	367	44	400	20	115
		%	11·5	88·5	10·3	89·7	9·9	90·1	14·8	85·2
Others	Rheumatoid arthritis	Cases	222	84	168	63	267	70	81	34
		%	72·5	27·5	72·7	27·3	75·6	24·4	70·4	29·6
	Not rheumatoid arthritis*	Cases	115	922	85	740	134	867	47	229
		%	11·1	88·9	10·3	89·7	13·4	86·6	17	83

* Not rheumatoid arthritis in this table includes patients diagnosed as "possible" and "probable" rheumatoid arthritis. This accounts for the high percentage of positives in this group.

In this table, in order to obtain the greatest accuracy, results on patients from the Rheumatism Research Unit have been used for all comparisons except those involving the "RA-test," for which the rather smaller numbers have made it necessary to include patients from all sources. The "likelihood ratios," values of which are given in Table IV, provide an index of the reliability of the relevant result in the diagnosis of rheumatoid arthritis. The likelihood ratio for a particular type of result is defined as:

$$\frac{\text{Probability of this result in patients with rheumatoid arthritis}}{\text{Probability of this result in patients without rheumatoid arthritis}}$$

In the absence of any other relevant knowledge about the patient the likelihood ratio expresses the odds in favour of rheumatoid arthritis for a patient showing this particular result. The more efficient a test, or combination of tests, the higher the likelihood ratio for a positive result and the lower the ratio for a negative result. In interpreting Table IV it must be noted that comparisons can only be made between figures in the same column, comparisons between figures in different columns not being valid. Consideration of the figures shows some superiority of the Rose-Waaler over both the bentonite and latex tests. The "RA-test" came out of the comparison a little better than the Rose-Waaler test. Bentonite/"RA-test" comparisons were too few to be worth recording in Table IV.

It can be seen from Table IV that for any pair of tests the result when the tests are in agreement is more reliable than the result of either of the tests singly, i.e., the likelihood ratios for double positives and negatives are respectively higher and lower than for either of the individual members of the pair.

In the great majority of instances in which the Rose-Waaler test was positive in the presence of a negative result with one or more of the other tests, the Rose-Waaler titre of the serum was low. The modal titre of all positive sheep cell tests was not less than 1,024, whereas the modal titre in cases giving discordant results was 256. This applied to false as well as true positive Rose-Waaler results. Conversely it was usual, though not invariable, for the positive results to be weak in the latex, bentonite, or "RA-test" in sera which gave a negative result in the Rose-Waaler test.

The Rose-Waaler test very occasionally shows a prozone effect, the agglutination being weaker, but never absent, in the lower than in the higher serum dilutions. It seemed possible therefore that, since only a single low serum dilution was being used for the factor II group of tests, prozoning in these tests might be responsible in some instances for unexplained negative results. Accordingly a considerable number of sera giving unexpectedly negative or weak results in the latex test were fully titrated out with latex. Although in a very few sera the particle

TABLE IV
LIKELIHOOD RATIOS IN FAVOUR OF A DIAGNOSIS OF RHEUMATOID ARTHRITIS

Rose-Waaler / Bentonite (590 cases)			Rose-Waaler / Latex (615 cases)			Bentonite / Latex (579 cases)			Rose-Waaler / "RA-test" (332 cases)			Latex / "RA-test" (313 cases)		
Both	+	17·1	Both	+	27·3	Both	+	23·5	Both	+	14·5	Both	+	14·3
Rose-Waaler	+	11·7	Rose-Waaler	+	12·8	Bentonite	+	11·1	Rose-Waaler	+	8·9	Latex	+	9·3
Bentonite	+	9·9	Latex	+	10·3	Latex	+	10·5	"RA-test"	+	10·4	"RA-test"	+	10·2
Both	−	0·170	Both	−	0·150	Both	−	0·170	Both	−	0·186	Both	−	0·150
Rose-Waaler	−	0·204	Rose-Waaler	−	0·184	Bentonite	−	0·229	Rose-Waaler	−	0·246	Latex	−	0·204
Bentonite	−	0·245	Latex	−	0·207	Latex	−	0·227	"RA-test"	−	0·235	"RA-test"	−	0·203

aggregation was more marked in higher serum dilution, in no instance was a positive result obtained at a higher dilution in the presence of a negative result at 1 in 20. It therefore seems that prozoning cannot explain a false negative reaction with the latex test.

Discussion

All three tests under trial have a 90% agreement with each other and with the Rose-Waaler test, although the latter comes out rather better from the comparison than the latex and bentonite tests. The proportion of equivocal results was also lowest for the Rose-Waaler test, although perhaps this is not an absolutely fair comparison because the Rose-Waaler test was always set up as a full titration, whereas only a single serum dilution was used for the other tests. It might appear from these results that there is not a great deal to choose between the four tests. However, ease of performance and of reading must also be taken into account.

The bentonite test is very elegant and the agglutination of bright blue particles is an unusual and pleasing sight, although considerable difficulty was found in obtaining suspensions of constant stability. Some preparations were almost spontaneously agglutinable, giving immediate agglutination with positive sera, but shortly afterwards giving a similar appearance in negative controls. It was therefore necessary to watch the tests carefully throughout the incubation period.

The tube latex test is very simple and in the majority of cases is easy to read. Minor degrees of aggregation, however, are less easy to be sure of and are more difficult to read than agglutination in corresponding serum dilutions in the Rose-Waaler test. The latex test, and, to a lesser extent, the bentonite test, is rather sensitive to raised serum globulin, and in the presence of hyperglobulinaemia the results are less reliable.

The " RA-test " has been found to be extremely satisfactory. It is quick to do, easy to read, and requires a minimum of apparatus and no centrifuging. The reagent is rather expensive. During this survey and since its completion, we have tried out a home-made fraction II/latex reagent, which has given results which have been almost identical with those of the " RA-test," although we have not been able to achieve a reagent which remains quite so homogeneous in negative serum as does the Hyland preparation. The " RA-test " or some modification of it seems to be the test of choice where time or facilities are limited or where the small number of sera to be tested makes the Rose-Waaler test uneconomical in time and effort.

Since there is an advantage (Table IV) in using two tests and evidence that the Rose-Waaler and the fraction II tests measure different substances (see below), it seems logical to use the Rose-Waaler in combination with one of the other tests. The " RA-test " or a modification of it is the simplest and most useful of the fraction II tests which we have tried out, and we are now regularly using our modification of it in conjunction with the Rose-Waaler test.

Finally, a word must be said on the reason for divergence of results between these tests. Some differences, though probably few, are due to variations in technique and reagents; for instance, occasional rheumatoid sera only give a positive Rose-Waaler test if the cells are sensitized to the very brink of agglutination (a condition not always attained), and in a very few the agglutinating property is considerably diminished by even the short period of inactivation. Similarly the fraction II tests must be at the mercy of the reagents and their sensitivity will vary according to the degree of denaturation and aggregation of the fraction II preparation used.

It is, however, probable that the fraction II and sensitized red cell tests are not indicating identical substances. Thus Heller, Jacobson, Kolodny, and Kammerer (1954) showed that absorption of a rheumatoid serum with sensitized red cells would remove the Rose-Waaler reacting property, but would leave almost unimpaired the ability to react with fraction II, whereas absorption with fraction-II-coated cells removed both properties. Also Greenbury (1956) showed that in elution experiments the amount of protein required to cause agglutination was much less than the quantity of rheumatoid factor estimated to be in the serum by some workers, and current work (Greenbury, unpublished) is tending to confirm this. More recently Lospalluto and Ziff (1959) have by chromatographic methods separated the rheumatoid factor into fractions which differ in their relative content of fraction-II-precipitating and

TABLE V

	Date	Rose-Waaler Titre	Bentonite	Latex
Patient A: Rheumatoid arthritis	12/57	32	−	−
	2/58	256	−	−
	4/58	64	−	−
	11/58	256	±	−
	1/59	256	−	−
Patient B: Rheumatoid arthritis	9/58	<2	±	±
	10/58	<2	−	+ +
	11/58	<2	−	+
	12/58	<2	−	+
Patient C: Unresolving pneumonia	12/57	<2	+	−
	1/58	<2	+	−
	3/58	<2	+	−

sensitized-cell-agglutinating substances. Further evidence of dissociation of properties of the rheumatoid factor is found in the present series; it is not uncommon for patients to maintain over long periods a constant pattern of reaction to the different tests (see Table V .

It may of course be that in patient C the reaction is not with fraction II but with a fraction of the patient's own serum which has been absorbed to the bentonite

It is not uncommon, early in the disease, for either the latex or Rose-Waaler test alone to be positive, the second test not becoming positive until later on. Similarly during remission one of the tests may become negative while the other remains positive. Further work is proceeding on the quantitative aspects of the sensitized red cell and fraction-II-combining properties of the rheumatoid factor.

It is worth noting how comparatively unreliable is the diagnosis given on laboratory request forms. This is well shown in Table III, where the results from the Rheumatism Research Centre and from other sources are compared. It is clear that laboratory request forms are not a suitable basis upon which to form conclusions concerning the accuracy of laboratory tests, at least where diagnosis of the disease in question is not straightforward.

I wish to thank Dr. A. G. S. Hill, Director of the Oxford Regional Rheumatism Research Centre, for clinical information concerning his patients, and, together with Dr. Hill, Dr. M. S. Good, and Dr. R. Spalding Smith, for their permission to use some of the material collected for our joint publication on the clinical significance of the Rose-Waaler test. I wish to thank also Dr. P. Armitage, of the Statistical Research Unit of the M.R.C., for his statistical help and advice.

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THE HAEMAGGLUTINATION TEST FOR TOXOPLASMA ANTIBODIES

BY

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Four hundred and forty-six patients' sera from a hospital blood transfusion department in Newcastle were examined for toxoplasma antibodies by a haemagglutination test.

Forty per cent. of the sera gave positive reactions, and an increase in the percentage of positive sera was found with increasing age, rising to over 60% at the age of 70 and over. A wide range of titres was demonstrated amongst positive cases, the highest titres approaching those obtained in two cases of active toxoplasmosis.

In a separate survey, no quantitative agreement was found between dye test titres and haemagglutination titres.

As knowledge of toxoplasmosis has increased, efforts have been made to improve and extend the range of specific diagnostic tests. At present, the test most commonly employed is the Sabin-Feldman dye test or one of its modifications. This is technically exacting to perform, and requires the presence of live toxoplasmas, as well as a supply of normal accessory factor serum which is often difficult to obtain. In the search for alternative methods, attempts have been made to develop a haemagglutination test for the presence of toxoplasma antibodies in serum. Bozdečh and Jíra (1958), using sensitized human group O Rh-negative cells, concluded that the test was less sensitive even than the complement-fixation reaction. Jacobs and Lunde (1957) and Lunde and Jacobs (1958), using the tanned red cell technique of Boyden (1951), obtained results which corresponded well with dye test titres, but stressed the need for further work before it could become an established technique.

In the present investigation, we have attempted to confirm the value of the test, using it, after suitable modifications, to determine the incidence of toxoplasma antibodies in a hospital population provided by recipients' sera in a blood transfusion department.

Materials and Methods

The antigen was prepared from the peritoneal exudate of mice inoculated four days previously with a strain of toxoplasma known to have been of constant virulence for mice over at least two years. The pooled exudate was centrifuged for 30 min. at 3,000 r.p.m. and the supernatant discarded. The sediment was washed twice in normal saline, and resuspended in phosphate buffered saline (see below) to the original volume of the exudate. The toxoplasmas were disrupted by ultrasonic vibration at 20 Kc for one hour, using a Mullard-M.S.E. disintegrator. Cellular debris was removed by centrifuging for 30 min. at 3,000 r.p.m. The supernatant constituted the neat antigen. It was tubed off in small amounts sufficient for each batch of tests, and stored at −20° C.

The haemagglutination test proper was based on the technique of Boyden and Sorkin (1955). Instead of sheep cells, human group O Rh-negative cells in citric acid-dextrose solution were used, as described by Roitt and Doniach (1958). Apart from their ready availability in hospital and freedom from contamination, their use avoided the necessity of absorbing large numbers of sera with species-different packed cells before the test. Occasionally cells were obtained freshly by venepuncture.

Normal saline buffered with a phosphate mixture, prepared according to Stavitsky (1954), was used at all stages of the test.

The serum-saline "stabilizer" used to suspend the sensitized cells and to dilute the test sera was 2% human AB Rh-positive serum in buffered saline. The serum was previously tested and found negative for toxoplasma antibodies.

The red cells were washed six times in saline and made up to a 2½% suspension in saline. The cells were tanned at 37° C. for 15 min. using 1 volume of 1/40,000 tannic acid (Merck) in saline. After a single wash in 2 volumes of saline, the cells were reconstituted to a 2½% suspension. One volume of suitably diluted antigen (see below) was added and sensitization carried out for approximately 15 min. at room temperature. The tanned sensitized cells were washed twice in 2 volumes of 0·5% serum-saline, and resuspended in 2% serum-saline to a 0·5% suspension. Cells sensitized in this way could not be relied upon to retain constant activity for more than one day unless 0·25% formalin was added to the serum-saline. If the formolized cells were left overnight before use, they were subsequently found to give constant titres with the same positive control serum for at least four days, and on one occasion were found to work satisfactorily for up to two weeks when stored at 4° C.

Haemagglutination was carried out in small rimless tubes (1¼ in. × ⅜ in.) placed at an angle of 50° to the horizontal as described by Boyden and Sorkin (1955), and set up in a row of 12 tubes for each test serum. New, unwashed tubes were found suitable and were discarded after use. Each tube received 0·2 ml. of sensitized cells. Test sera were diluted 1 in 10 in 2% serum-saline. Serum, 0·2 ml., was added to the first tube, and serial twofold dilutions made along the row, giving a range of serum dilutions 1 in 10 to 1 in 20,480. The end-point was taken as the last tube in which cells adhered appreciably to the back of the tube. The results could usually be read approximately two hours after setting up the test at room temperature, and were expressed as the reciprocal of the final dilution. If all sera were previously inactivated at 58° C. for 15 min., and the racks stored at 4° C. overnight, the results could conveniently be read the following morning.

Multiple negative and positive control sera were used in the test. The titres obtained were arbitrarily related to a standard positive serum (dye test titre 1 in 70) used throughout the investigation. The strength of antigen employed was checked and if necessary adjusted for each batch of cells so that the titre given by this serum fell within the range six to eight tubes. This standard was conveniently chosen so that the highest titres encountered in test sera rarely exceeded 12 tubes.

When testing large numbers of sera, a preliminary screening test was carried out to a three-tube dilution to separate positive from negative sera. In this way, positive sera collected over several weeks could be taken to their final titres in one large batch, using a single preparation of cells, thereby obtaining the most uniform conditions for quantitative testing.

Results

Four hundred and forty-six sera obtained during 1959 from the hospital blood transfusion department of the Royal Victoria Infirmary, Newcastle, were examined, and of these 177 (40%) gave positive

TABLE I
HAEMAGGLUTINATION TITRES AT VARIOUS AGES

Titres	Age in Years							
	0–9	10–19	20–29	30–39	40–49	50–59	60–69	70+
Negative	31	42	45	32	36	39	22	22
20–40	2	2	4	1	3	1	8	6
80–320	1	6	9	16	13	14	17	23
640–2,560	1	6	6	5	7	8	8	6
5,120–20,480	0	1	0	0	1	0	0	1
20,480	0	0	0	1	0	0	0	0

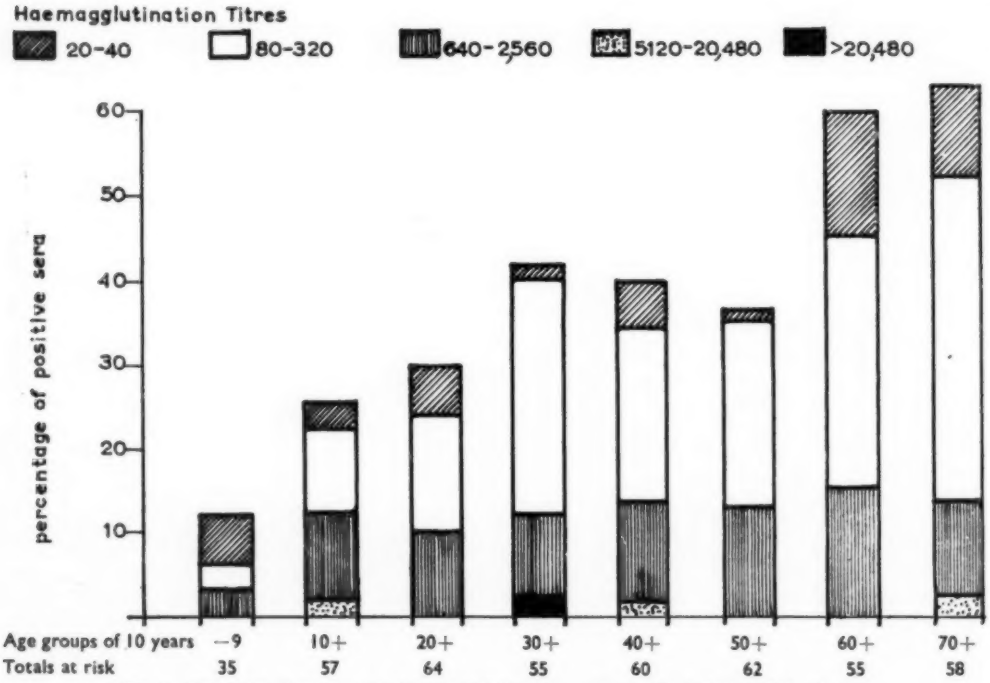

FIG. 1.—Percentages of positive sera demonstrated in various age groups by the haemagglutination test.

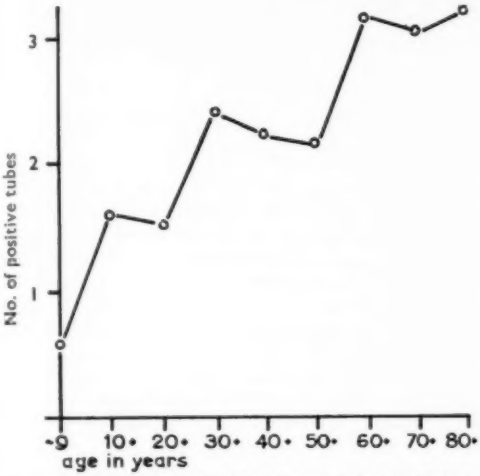

FIG. 2.—Average number of positive tubes per patient at risk in the various age groups.

reactions to a titre of 20 or more. Roughly equal numbers of sera were represented in the various age groups, and the absolute numbers and percentages of positive sera in the groups are given in Table I and Fig. 1. Sera from children under the age of 6 months were excluded from the series, to eliminate the possibility of maternal transfer of antibodies.

The incidence of positive sera appeared to rise in step-like fashion from 11% in the age group 0–9 years to 62% in patients over the age of 70. The same tendency is shown in Fig. 2, which expresses the average number of positive tubes per patient at risk in each age group. The highest titres are not encountered in any particular age group. The highest titre in the series, 40,960, was given by a serum from a patient aged 35, whilst a titre of 10,240 was given by a serum from a 10-year-old child.

Forty-three per cent. of 234 sera from females were positive compared with 39% of 186 sera from males.

The various blood groups were normally represented among the positive sera.

In a separate study, attempts were made to correlate haemagglutination titres with dye test titres. Of 73 sera received from source A, 17 gave a negative dye test (D.T.) of less than 1 in 4, and a negative haemagglutination test (H.T.); 35 gave a positive D.T. with a positive H.T.; 20 gave a positive D.T. with a negative H.T., but only one serum gave a negative D.T. with a positive H.T. The highest D.T. titre of a serum giving a negative H.T. was 1 in 110. No quantitative relationship was found when D.T. titres were plotted against H.T. titres (Fig. 3).

Of 25 sera from another source B, all with positive D.T. titres of 1 in 10 or more, 23 gave a positive

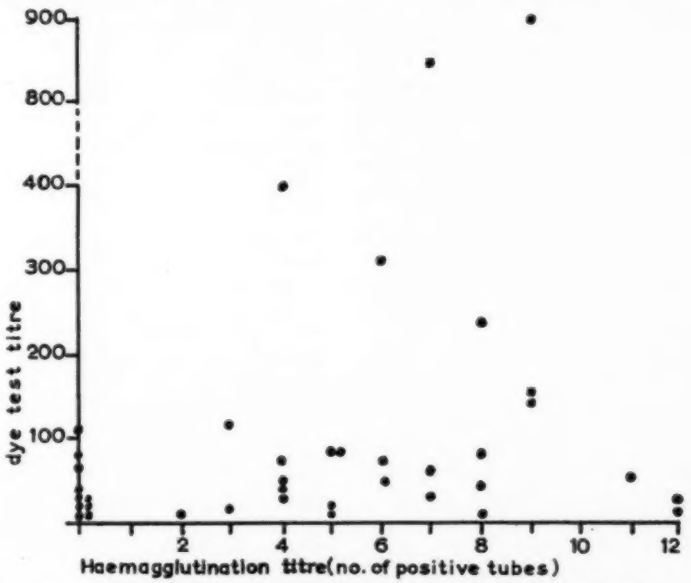

FIG. 3.—Sera from source A: dye test titres and haemagglutination titres compared.

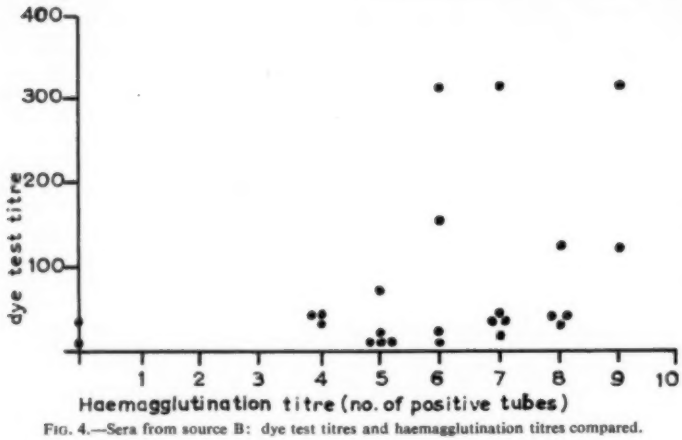

FIG. 4.—Sera from source B: dye test titres and haemagglutination titres compared.

H.T., but there was again no quantitative correlation between D.T. titres and H.T. titres (Fig. 4). The two sera found negative by the H.T. had D.T. titres of 1 in 10 and 1 in 40.

Discussion

In trying to repeat the haemagglutination technique of Jacobs and Lunde, we were unable to produce potent antigens by the lysis of toxoplasmas in distilled water. Indeed, the organisms appeared to be surprisingly resistant to disruption even by repeated freezing and thawing. The method of ultrasonic disintegration finally adopted was suggested by Jacobs and Lunde (1957) themselves, and was previously used by Gronroos and Westphal (quoted by Cooney, Kimball, and Bauer, 1958), and by Hook and Faber (1957), who used this method in their studies on the nature of toxoplasma antigens. Incidentally we were able to confirm the finding of these last two authors that the fraction obtained from crude antigen by precipitation with ammonium sulphate at 30% saturation retains antigenic potency since it is able to sensitize red cells in the haemagglutination reaction.

Jacobs and Lunde considered that their antigens remained stable for periods of between five and 17 months when stored at −15° C. The antigens prepared by ultrasonic vibration showed a continuous loss of potency on storage at −20° C. over a period of eight weeks or less. Toxoplasmas in buffered saline stored for long periods at −20° C. and then disintegrated were found unsuitable for antigen preparation.

Although sheep cells worked satisfactorily in the test, it was found more convenient to use human cells for reasons already given. It was found unnecessary to alter the *p*H from 7.2 to 6.4 during sensitization. The sloped tube technique was used, partly to overcome the difficulties of interpreting characteristic haemagglutination patterns at the bottom of wide-bore tubes, and partly with the object of increasing, if possible, the sensitivity of the test.

Jacobs and Lunde use their antigens at such a concentration that the haemagglutination titre of a positive control serum is the same as its dye test titre. They find close agreement as to positivity and titre of test sera using both methods, to within a four-fold difference in haemagglutination titre in over 90% sera. We have not been able to confirm this with our modified technique. In a series of 73 sera, 20 with a positive dye test gave a negative haemagglutination test, although, as discussed below, the haemagglutination reaction is possibly more sensitive in detecting positive sera in a population survey. This discrepancy may perhaps be due to deterioration of sera following storage and repeated investigation, and it appears advisable in future work to carry out both tests simultaneously, if possible in the same department. No quantitative relationship was shown between the results of both tests, and consequently no attempt has been made to standardize one test in terms of the other. There is no reason why these two tests should give parallel results, any more than do the complement-fixation reaction and the dye test, and it cannot be assumed that the dye test and haemagglutination test are measuring the same antibody components.

Beverley and Beattie (1954), using a modification of the Sabin-Feldman dye test in a survey of 581 sera from "normal" persons in Sheffield, found that infection was rare in early life, but that over the age of 20 years approximately 25% of the population showed antibodies in their sera. There was no further rise in incidence over the age of 20. The percentage of positive sera in the present investigation is considerably higher, and continues to rise throughout life. However, we have examined fewer sera, drawn from a hospital population in a different part of the country, so that the two surveys cannot be directly compared. As it was possible that our higher incidence could be accounted for by the

inclusion of active cases of toxoplasmosis, clinical details were sought from those patients giving the highest titres. Except for the instance of an 80-year-old woman with a haemagglutination titre of 5,120, admitted to hospital with a haematemesis, who was found to have an ulnar neuritis and blindness in one eye—both conditions which might conceivably have been due to toxoplasmosis—there was nothing to suggest current infection. In any case, patients requiring urgent blood transfusion or about to undergo planned surgical procedures were primarily suffering from a totally dissimilar condition.

Sera from two patients with clinically active toxoplasmosis, and not included in the series, both gave haemagglutination titres of 160,000. The upper range of titres demonstrated in the present survey closely approaches this high value, so that it may not prove possible to define the upper limit of normal values for this method. It does not appear that a given titre can be interpreted any more significantly in conjunction with the patient's age, since high titres may be encountered at all ages. This problem, which is essentially that of distinguishing between present and past infection, is of course met when interpreting dye test titres.

The haemagglutination test for toxoplasma antibodies is at any rate seen to be a safe and practical test. It is extremely sensitive, and indeed might be made less sensitive with advantage. If stable antigens can be prepared and distributed, the test should lend itself to greater standardization than is possible with the dye test, and warrants further investigation.

We are indebted to Mr. E. C. Elliott, F.I.M.L.T., for skilled technical assistance; to Professor C. P. Beattie and Dr. I. A. B. Cathie for providing sera of known dye test titre; and to Dr. G. B. Ludlam for his helpful comments on the paper.

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PENICILLIN RESISTANCE IN STAPHYLOCOCCI ISOLATED IN A CASUALTY DEPARTMENT

BY

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The results of a series of antibiotic resistance tests on 118 strains of *Staphylococcus aureus* isolated from patients attending a casualty department are recorded. Forty-six per cent. of the strains were resistant to penicillin, 12% to streptomycin, 7.5% to tetracycline, and small numbers to the other antibiotics. It is suggested that tetracycline should be used in those infections which are sufficiently severe to merit antibiotic treatment.

The rapid progress of medical science is, in one instance at least, paralleled by the rapidity of the evolutionary progress of the antagonist, the pyogenic staphylococcus. When penicillin was first added to the range of weapons available for the fight against infection its advent was hailed as the dawn of a new era in the treatment of staphylococcal infections, which were one of the major problems of the surgeon. Initially this hope was fulfilled and staphylococci became less feared in surgical practice. Unfortunately, however, strains of staphylococci capable of producing an enzyme which destroyed penicillin soon began to appear. By 1951, only seven years after the introduction of penicillin into routine hospital practice, Rountree (1951) was able to find a high proportion of resistant strains in infections treated at a hospital out-patient department and three years later the same author (Rountree, Freeman, and Barbour, 1954) found that three-quarters of the strains of staphylococci isolated from hospital in-patients were resistant to this antibiotic. Since that time the situation has materially deteriorated; in 1959 Goodier and Parry found that over 80% of the strains of staphylococci isolated from in-patients were resistant to penicillin, as were over half the strains isolated from out-patients.

It is, nevertheless, still common practice for penicillin to be used freely in the therapy of relatively minor infections, and Lowden (1955), in a textbook on the casualty department, refers only to penicillin resistance in the case of infections in hospital staffs and patients recently in hospital.

The present paper comprises a study of the antibiotic resistance of strains of *Staphylococcus aureus* isolated from minor infections of skin and subcutaneous tissue of patients attending the casualty department of the David Lewis Northern Hospital, Liverpool, between April and June, 1959. Swabs were taken at the first attendance or, in the case of closed lesions, at the time of incision. The series is, as far as possible, composed of consecutive cases all of which were seen for the first time with the infection to which the study relates. Infections among members of the hospital staff and in-patients attending other departments were excluded from the series.

Method

The swabs were inoculated on to blood agar plates and were incubated at 37° C. for 24 hours. Colonies morphologically resembling *Staphylococcus pyogenes*, usually in pure culture, were identified by Gram staining and were tested for coagulase activity by the

TABLE I

RESULTS OF TESTS ON 118 STRAINS OF STAPHYLOCOCCI

		Open Lesion	Closed Lesion	Total
Penicillin ..	Resistant	21 (54%)	33 (43%)	54 (46%)
	Sensitive	18 (46%)	46 (57%)	64 (54%)
Streptomycin	Resistant	5 (13%)	9 (11%)	14 (12%)
	Sensitive	34 (87%)	70 (89%)	104 (88%)
Tetracycline	Resistant	3 (8%)	6 (7·5%)	9 (7·5%)
	Sensitive	36 (92%)	73 (92·5%)	111 (92·5%)
Chloram-phenicol	Resistant	— —	— —	— —
	Sensitive	39 (100%)	79 (100%)	118 (100%)
Erythromy-cin	Resistant	— —	— —	— —
	Sensitive	39 (100%)	79 (100%)	118 (100%)
Oleandro-mycin	Resistant	1 (2%)	2 (2·5%)	3 (2·5%)
	Sensitive	38 (98%)	77 (97·5%)	115 (97·5%)
Novobiocin	Resistant	1 (2%)	1 (1·2%)	2 (1·7%)
	Sensitive	38 (98%)	78 (98·8%)	116 (98·3%)

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TABLE II
INCIDENCE OF PENICILLIN-RESISTANT STRAINS

		Open Lesion	Closed Lesion	Total
Not on penicillin	Resistant	12 (46%)	24 (36%)	36 (39%)
	Sensitive	14 (54%)	42 (64%)	56 (61%)
On penicillin	Resistant	9 (69%)	9 (69%)	18 (69%)
	Sensitive	4 (31%)	4 (31%)	8 (31%)

slide technique of Williams and Harper (1946) using human plasma. All coagulase-positive staphylococci were tested for sensitivity to penicillin, streptomycin, tetracycline, erythromycin, oleandomycin, and novobiocin. The organisms were subcultured on to nutrient agar so as to produce a profuse growth and either "sentest" tablets (Evans) or "multodisks" (Oxoid) were placed on the surface of the medium before incubation. Sensitive organisms showed a zone of inhibition round the centre of the tablet or disc of at least 1 cm. in diameter; resistant organisms showed a profuse growth right up to the edge of the disc.

One hundred and eighteen strains of staphylococci were tested by this method and the results are summarized in Table I. Twenty-six of the patients from whom these organisms had been isolated had been treated with penicillin in all cases before the swabs were taken and before bacterial sensitivity tests had been carried out. In these the severity of the local infection and the presence of evidence of extension of the infective process along the lymphatic channels indicated some form of therapy in addition to surgical intervention and local applications. In Table II the incidence of penicillin-resistant strains is compared in the two groups, those receiving and those not receiving penicillin.

Discussion

There has been over the years a steady increase in the proportion of strains of penicillin-resistant staphylococci from infected lesions in hospitals. Outside the hospital the process has been less rapid, partly because the majority of staphylococci either live in a state of precarious symbiosis with their hosts or cause minor infections not deemed worthy of the antibiotic sledge-hammer, and partly because cross-infection with drug-resistant strains is less likely to occur. Nevertheless in the last 10 years reports have appeared which indicate that the incidence of penicillin-resistant infection among hospital out-patients is steadily increasing (Fig. 1).

We are tolerably certain that the infections which caused the patients in the present investigation to seek treatment in hospital were contracted in the course of their normal daily lives, and that the organisms cannot be regarded as hospital staphylococci. In 26 instances the patients had been treated with penicillin and the incidence of penicillin-resistant infection in this group was significantly higher than in the remainder.

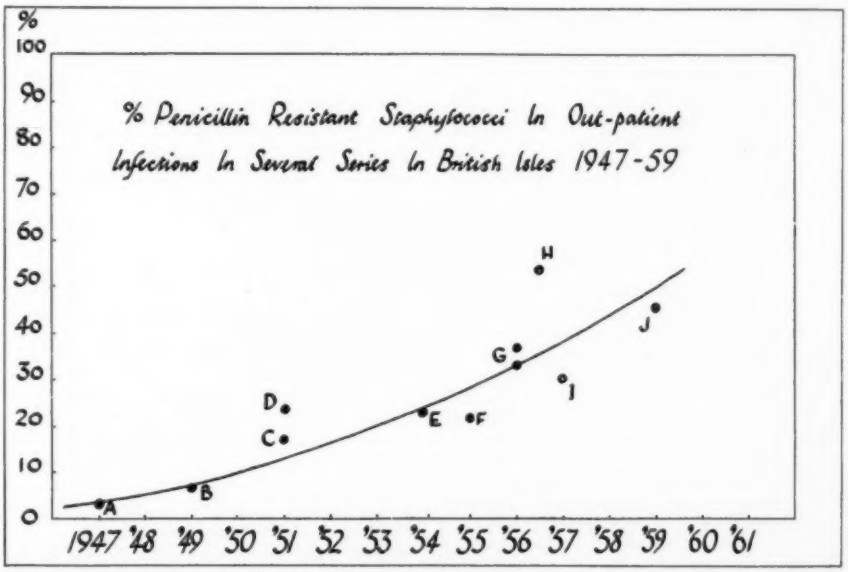

FIG. 1.—The increase in penicillin-resistant staphylococci in out-patient infections in a British series 1947–59. A, Bolton *et al.* (1947); B, Griffiths *et al.* (1949); C, Birnstingl *et al.* (1952); D, Summers (1952); E, Roodyn (1954); F, Rees *et al.* (1955); G, Fairbrother (1956); H, Goodier and Parry (1959); I, Buhr and Scott (1959); J, present series.

The alternative antibiotics available for the treatment of staphylococcal infections are streptomycin, chloramphenicol, the tetracyclines, the erythromycin group, and novobiocin. The two latter should be reserved for staphylococcal infection resistant to other antibiotics, and the ease with which all bacteria, including staphylococci and tubercle bacilli, become resistant to streptomycin make its extensive use in casualty departments undesirable, so that the only alternative to penicillin is one of the tetracycline antibiotics.

We therefore urge a change in the treatment of staphylococcal infections in the casualty department and in general practice. First, antibiotics should be eschewed as far as possible and the purulent lesions should be treated on general surgical lines. Secondly, if the infection is of such severity and has spread so far that antibiotic therapy is indicated, bacteriological examination and sensitivity tests should precede the administration of any antibiotic.

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THE SPECIFICITY OF THE SABIN-FELDMAN DYE TEST WITH REFERENCE TO PROTOZOAL INFECTIONS

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Infections of *Eimeria stiedae* in normal and splenectomized rabbits and of *Leishmania enriettii* in normal rabbits and guinea-pigs and in splenectomized rabbits did not produce dye test antibodies. Guinea-pigs injected with *Atoxoplasma sp.* and rabbits immunized with cultures of *Crithidia fasciculata* were found to be negative for dye test antibodies. Mice which had been infected with *Trypanosoma cruzi* did not show dye test antibodies. All these animals, except one guinea-pig infected with *Leishmania enriettii*, were negative in the complement-fixation test for toxoplasmosis using the egg antigen. These findings are discussed in the light of previous reports.

Westphal and Knüttgen (1950) were the first to examine the specificity of the dye test (Sabin and Feldman, 1948) with reference to protozoal infections. They could not obtain a positive dye test in the sera of 14 patients suffering from tertian malaria or in the sera of two patients, one with amoebic dysentery and the other with mucocutaneous leishmaniasis. Westphal (see Mühlpfordt, 1951; Thalhammer, 1957) could not find dye test antibodies in infections of trypanosomes. However, Mühlpfordt (1951) was able to demonstrate dye test antibodies in rabbits, rats, and a golden hamster which had been experimentally infected with *Sarcocystis*. In sheep harbouring *Sarcocystis tenella* he found antibodies which were ascribed to the presence of these infections. Dye test antibodies were also detected in sheep infected with *Sarcocystis tenella* by Awad and Lainson (1954a, b) and by Awad (1954a) using his modified dye test. However, Moscovici (1953, 1954) could not find any antigenic relationship between *Sarcocystis* and *Toxoplasma*. Michalzik (1953) observed a high percentage of dye test antibodies in women harbouring infections of *Trichomonas vaginalis*. This was experimentally confirmed by Awad (1954b) in mice immunized with bacteria-free, living *T. vaginalis*. He also reported the presence of dye test antibodies in mice infected with *Trypanosoma cruzi*. Cathie (1955a, b) refuted some of these findings. Frenkel (1953) could not demonstrate any antigenic relationship between *Toxoplasma* and *Besnoitia jellisoni*. Since contradictory reports had been made on the production of dye test antibodies due to protozoal infections, it was felt necessary to undertake a detailed study to verify these observations.

MATERIALS AND METHODS

The following procedures were adopted throughout the study except where otherwise stated. A sample of blood was obtained from each experimental animal before infection. The bleeding of the experimental animals was carried out once a week, by cardiac puncture without any anaesthetic. About 8 ml. of blood was obtained at a time. It was allowed to clot at room temperature and the clot separated where necessary by means of a sterile platinum loop. The specimens were refrigerated overnight and centrifuged in the morning for 15 minutes at full speed, using an M.S.E. bench centrifuge. The separated serum was bottled in Bijou bottles and stored in the deep freeze at −17° C. till required for study. The period of storage was no longer than four months.

As accessory factor sera human sera were used from three donors who had been previously tested for this purpose and found suitable. The accessory factor sera were stored over dry ice and only thawed just before use to minimize the loss of the accessory factor content. The period of such storage never exceeded four months.

The *Toxoplasma* suspension was prepared by adding 0.1 ml. of a 1:500 solution of heparin in saline to each of the peritoneal exudates from albino mice 6 to 8 weeks old that had been injected three days previously with 1:10 suspension of peritoneal fluid (each high-power microscopic field containing about 15 to 25 parasites) from mice harbouring three-day infections of the " RH " strain of *Toxoplasma gondii*. Those suspensions showing a volume of 0.5 to 1.0 ml were selected first. These were then examined under the microscope for adequate numbers of *Toxoplasma*. Only exudates which were free from bacterial contaminants and which showed over 100 *Toxoplasma* per high-power microscopic field were finally selected for use in the test. An aqueous solution of 0.25% methylene blue was used as the indicator and was prepared in the carbonate-borate buffer, as recom-

mended by Sabin and Feldman (1948), a week earlier than required. This solution was used during the subsequent week and then discarded.

The freshly thawed test sera were inactivated for half an hour in a water-bath at 56° C. Four-fold serial dilutions were carried out up to an original dilution of 1:256 using 0.85% saline as diluent. To 2 vol. of each of the serum dilutions were added 4 vol. of the freshly thawed accessory factor serum and 1 vol. of the heparinized *Toxoplasma* suspension. A suspension control was instituted for the *Toxoplasma* suspension from each mouse. This was prepared by adding 2 vol. of saline and 4 vol. of the accessory factor serum to 1 vol. of the *Toxoplasma* suspension. A known positive serum control was added to detect the presence of a sufficient amount of accessory factor in the accessory factor serum. The positive serum was obtained from a rabbit previously injected with the avirulent "RB25" strain of *Toxoplasma*. The contents of the tubes were mixed thoroughly by shaking the tube racks gently. The tubes were incubated for 45 minutes in a water-bath at 37° C. At the end of this period 1 vol. of alkaline methylene blue was added to each tube and mixed thoroughly as before. The tubes were again incubated for a further five minutes. The readings were made immediately after removal from the water-bath. Whenever the readings could not be carried out immediately the tubes were refrigerated soon after removal from the water-bath. However, all tests were always read before the elapse of two hours after the addition of the methylene blue. A batch of results was accepted only if the accessory factor serum control showed less than 10% modification and the positive control worked faultlessly. The titre was taken as the highest original dilution showing more than 50% modification of the extracellular *Toxoplasma*.

The complement fixation test of Warren and Russ (1948) was also carried out on the sera with some of the modifications as adopted by Lainson (1955). The sera for the complement fixation test were inactivated for half an hour in a water-bath at 56° C. They were serially diluted in two-fold dilutions up to a maximum dilution of 1:16, using 0.85% saline. One volume of antigen containing 4 units and 1 vol. of the complement diluted to contain 4 M.H.D. per volume were added to 1 vol. of each of the serum dilutions. An antigen control obtained from uninfected chick chorio-allantoic membranes and an anti-complement control were instituted for each serum and known positive and negative serum controls were added to each batch of tests. The contents of the tubes were mixed thoroughly by shaking the racks. They were incubated for one hour in a water-bath at 37° C. At the end of this period 2 vol. of a sensitized sheep cell suspension prepared by mixing equal volumes of a haemolysin solution containing 6 M.H.D. of haemolysin and a 5% sheep cell suspension and incubating together for half an hour at 37° C. were added. The contents of the tubes were mixed as before and reincubated for another half an hour. The results of the tests were read only if the controls had worked faultlessly by this time.

EXPERIMENTS

Experiment RBC: Dye Test Antibodies in Rabbits Infected with *Eimeria stiedae*

Twenty-four normal rabbits, 11–13 weeks old, were bled in two batches of 12 each. They were then fed with a large quantity of sporulated oocysts of *Eimeria stiedae* obtained from the faeces of infected rabbits. The oocysts were concentrated by salt flotation and sporulated in 2% sodium dichromate solution. The sodium dichromate was washed off by repeated centrifuging in water before feeding the rabbits. Weekly samples of blood were drawn from the rabbits as before, up to a total of eight bleedings or the prior death of the rabbit. Samples of blood were also taken after death by cutting open the superior and inferior venae cavae in a few.

The tests were carried out as detailed earlier on inactivated and non-inactivated sera. Complement-fixation tests were carried out on inactivated sera.

Results.—These are set out in Table I. Two rabbits died at the first cardiac puncture. Fourteen rabbits died of coccidiosis by the third week, five rabbits by the fourth week, two rabbits by the fifth week, and one rabbit survived the infection and was sacrificed later. Three rabbits did not show any dye test titres at all. The rest had dye test titres ranging from 1 to 1:16. The titres fluctuated in the same rabbit between these limits. There was a slight difference in titre between the inactivated sera and the corresponding non-inactivated ones, but this difference was negligible, being of the order of one dilution. All rabbits were negative for complement fixing antibodies.

Experiment RBCS: Dye Test Antibodies in Splenectomized Rabbits Infected with *Eimeria stiedae*

Twenty-four rabbits, 10–12 weeks old, were splenectomized using standard techniques. About four weeks after the last splenectomy, they were bled in two batches and fed with a smaller dose of sporulated oocysts of *E. stiedae* than in the previous experiment. Weekly samples of blood were drawn. Dye tests and complement fixation tests were carried out on inactivated sera. Dye tests were also carried out on non-inactivated sera.

Results.—The results are summarized in Table II. Two rabbits died at the end of the seventh week. The rest survived the infection, probably due to being older rabbits than those in the previous experiment and also being infected with a smaller dose. Two rabbits were completely negative in the dye test. The rest showed titres ranging from 1 to 1:16. As before there was a slight difference of titre in the results of the inactivated and non-inactivated sera, but this was not significant. The complement fixation tests were negative.

Experiment RBL: Dye Test Antibodies in Rabbits Injected with *Leishmania enrietii* into Tips of Noses

Twenty-four rabbits, 6–8 weeks old, were bled in two batches and injected with *Leishmania enriettii* obtained from lesions of previously infected guinea-pigs. Weekly

TABLE I

DYE TEST ANTIBODIES IN NORMAL RABBITS INFECTED WITH *EIMERIA STIEDAE*

Rabbit No.	Test	Pre-inoculation Sera	1	2	3	4	5	6	7	8
RBC/1	DT/I	0	1 : 16	1 : 4	1 : 4					
	DT/N	1 : 4	1 : 16	1 : 4	1 : 4					
	CFT	0	0	0	0					
RBC/2	DT/I	0								
	DT/N	1 : 4								
	CFT	0								
RBC/3	DT/I	0	1 : 4	1 : 4	1 : 16	1 : 4				
	DT/N	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16				
	CFT	0	0	0	0	0				
RBC/4	DT/I	0	0	0	0					
	DT/N	1 : 4	1 : 4	1 : 4	1 : 4					
	CFT	0	0	0	0					
RBC/5	DT/I	0	1 : 4	0	0					
	DT/N	0	1 : 4	0	1 : 4					
	CFT	0	0	0	0					
RBC/6	DT/I	1 : 4	1 : 4	1	1					
	DT/N	1 : 16	1 : 16	1 : 4	1 : 4					
	CFT	0	0	0	0					
RBC/7	DT/I	1 : 4	1 : 4	1 : 4	1					
	DT/N	1 : 16	1 : 16	1 : 16	1 : 4					
	CFT	0	0	0	0					
RBC/8	DT/I	1 : 4	1 : 4	0	0					
	DT/N	1 : 4	1 : 4	1 : 4	0					
	CFT	0	0	0	0					
RBC/9	DT/I	1	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	DT/N	1 : 4	1 : 16	1 : 16	1 : 16	1 : 16	1 : 4	1 : 4	1 : 4	1 : 4
	CFT	0	0	0	0	0	0	0	0	0
RBC/10 ..	DT/I	1	1 : 4	1 : 4	0					
	DT/N	1 : 4	1 : 16	1 : 16	1 : 16					
	CFT	0	0	0	0					
RBC/11 ..	DT/I	0	0	0	0					
	DT/N	0	0	0	0					
	CFT	0	0	0	0					
RBC/12 ..	DT/I	0	1 : 4	0	0					
	DT/N	1 : 4	1 : 16	1 : 16	1 : 4					
	CFT	0	0	0	0					
RBC/13 ..	DT/I	1 : 4	1 : 16	1 : 4	1					
	DT/N	1 : 16	1 : 16	1 : 16	1 : 4					
	CFT	0	0	0	0					
RBC/14 ..	DT/I	1 : 4	1	1 : 4	1 : 4					
	DT/N	1 : 4	1 : 4	1 : 16	1 : 4					
	CFT	0	0	0	0					
RBC/15 ..	DT/I	1 : 4	1	1 : 4	1 : 4	1				
	DT/N	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4				
	CFT	0	0	0	0	0				
RBC/16 ..	DT/I	1	1 : 4	1 : 4	1 : 4	1 : 4				
	DT/N	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4				
	CFT	0	0	0	0	H				
RBC/17 ..	DT/I	0	0	0	0	0				
	DT/N	0	0	0	0	0				
	CFT	0	0	0	0	H				
RBC/18 ..	DT/I	1	1 : 4	1 : 4	1 : 4					
	DT/N	1 : 4	1 : 4	1 : 4	1 : 4					
	CFT	0	0	0	0					
RBC/19 ..	DT/I	1								
	DT/N	1 : 4								
	CFT	0								
RBC/20 ..	DT/I	1 : 4	1 : 4	1 : 4	1					
	DT/N	1 : 4	1 : 4	1 : 16	1 : 4					
	CFT	0	0	0	0					
RBC/21 ..	DT/I	1	0	0	1 : 4	0	0			
	DT/N	1 : 4	0	1 : 4	1 : 4	1 : 4	1 : 4			
	CFT	0	0	0	0	0	0			
RBC/22 ..	DT/I	0	0	0	0					
	DT/N	0	0	0	0					
	CFT	0	0	0	0					
RBC/23 ..	DT/I	1	0	1 : 4	0	0	0			
	DT/N	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4			
	CFT	0	0	0	0	0	0			
RBC/24 ..	DT/I	1 : 4	0	1 : 4	0	0				
	DT/N	1 : 4	0	1 : 4	0	0				
	CFT	0	0	0	0	0				

I = inactivated sera. N = non-inactivated sera. H = haemolysed sera. 1, 2, 3, etc. = weekly sera obtained after infection. DT = dye test. CFT = complement-fixation test.

TABLE II

DYE TEST ANTIBODIES IN SPLENECTOMIZED RABBITS INFECTED WITH *EIMERIA STIEDAE*

Rabbit No.	Test	Pre-inoculation Sera	1	2	3	4	5	6	7	8
RBCS/1 ..	DT/I	0	1	0	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	
	DT/N	1 : 4	1 : 4	1 : 16	1 : 16	1 : 16	1 : 4	1 : 16	1 : 4	
	CFT	0	0	0	0	0	0	0	0	
RBCS/2 ..	DT/I	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	DT/N	1 : 16	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	CFT	0	0	0	0	0	0	0	0	0
RBCS/3 ..	DT/I	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	DT/N	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 4	1 : 4	1 : 4	1 : 4
	CFT	0	0	0	0	0	0	0	0	0
RBCS/4 ..	DT/I	0	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	DT/N	1 : 4	1 : 4	1 : 4	1 : 4	1 : 16	1 : 4	1 : 4	1 : 4	1 : 4
	CFT	0	0	0	0	0	0	0	0	0
RBCS/5 ..	DT/I	1 : 4	1 : 4	1 : 16	1 : 16	1 : 16	1 : 16	1 : 4	1 : 4	1 : 4
	DT/N	1 : 4	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16
	CFT	0	0	0	0	0	0	0	0	0
RBCS/6 ..	DT/I	1 : 4	1	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	DT/N	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 4	1 : 4	1 : 4	1 : 4
	CFT	0	0	0	0	0	0	0	0	0
RBCS/7 ..	DT/I	0	0	0	0	0	0	0	0	0
	DT/N	0	0	0	0	0	0	0	0	0
	CFT	0	0	0	0	0	0	0	0	0
RBCS/8 ..	DT/I	0	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	DT/N	0	1 : 4	1 : 4	1 : 4	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16
	CFT	0	0	0	0	0	0	0	0	0
RBCS/9 ..	DT/I	0	0	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	DT/N	1 : 4	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16
	CFT	0	0	0	0	0	0	0	0	0
RBCS/10 ..	DT/I	0	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	DT/N	0	1 : 16	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	CFT	0	0	0	0	0	0	0	0	0
RBCS/11 ..	DT/I	0	0	0	0	0	0	0	0	0
	DT/N	0	1 : 4	1 : 4	1 : 4	0	0	1 : 4	1 : 4	1 : 4
	CFT	0	0	0	0	0	0	0	0	0
RBCS/12 ..	DT/I	0	0	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	DT/N	0	0	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	CFT	0	0	0	0	0	0	0	0	0
RBCS/13 ..	DT/I	1 : 4	1	1 : 16	1 : 4	1 : 4	1	1	1	
	DT/N	1 : 16	1 : 4	1 : 16	1 : 4	1 : 16	1 : 4	1 : 4	1 : 4	
	CFT	0	0	0	0	0	0	0	0	
RBCS/14 ..	DT/I	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	DT/N	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 4	1 : 4	1 : 4	1 : 4
	CFT	0	0	0	0	0	0	0	0	0
RBCS/15 ..	DT/I	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	DT/N	1 : 16	1 : 16	1 : 16	1 : 16	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	CFT	0	0	0	0	0	0	0	0	0
RBCS/16 ..	DT/I	1	1 : 4	1 : 4	1 : 4	0	1 : 4	1 : 4	1 : 4	1 : 4
	DT/N	1 : 4	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16
	CFT	0	0	0	0	0	0	0	0	0
RBCS/17 ..	DT/I	0	0	0	0	0	0	0	0	0
	DT/N	0	0	0	0	0	0	0	0	0
	CFT	0	0	0	0	0	0	0	0	0
RBCS/18 ..	DT/I	0	1 : 4	0	1 : 4	0	1 : 4	1 : 4	1 : 4	1 : 4
	DT/N	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	CFT	0	0	0	0	0	0	0	0	0
RBCS/19 ..	DT/I	0	0	1	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	DT/N	0	0	1 : 4	1 : 16	1 : 16	1 : 4	1 : 4	1 : 16	1 : 16
	CFT	0	0	0	0	0	0	0	0	0
RBCS/20 ..	DT/I	1 : 4	1 : 4	1 : 4	1 : 4	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16
	DT/N	1 : 16	1 : 16	1 : 4	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16
	CFT	0	0	0	0	0	0	0	0	0
RBCS/21 ..	DT/I	1 : 4	1	1	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	DT/N	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	CFT	0	0	0	0	0	0	0	0	0
RBCS/22 ..	DT/I	0	0	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	DT/N	1 : 16	1 : 4	1 : 16	1 : 16	1 : 4	1 : 16	1 : 16	1 : 16	1 : 16
	CFT	0	0	0	0	0	0	0	0	0
RBCS 23 ..	DT/I	1 : 4	1 : 4	1 : 4	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16
	DT/N	1 : 4	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16
	CFT	0	0	0	0	0	0	0	0	0
RBCS/24 ..	DT/I	0	0	1 : 16	1 : 16	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	DT/N	1 : 4	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16	1 : 16
	CFT	0	0	0	0	0	0	0	0	0

I = inactivated sera. N = non-inactivated sera. H = haemolysed sera, 1, 2, 3, etc. = weekly sera obtained after infection. DT = dye test. CFT = complement fixation test.

samples of blood were obtained. Dye tests were carried out on inactivated sera only. Complement fixation tests were also carried out.

Results.—The results are set out in Table III. None of the rabbits was infected with *L. enriettii* macroscopically. One rabbit died by the end of the first week, one by the end of the second week, two by the end of the seventh week, and the rest were all sacrificed at the end of the eighth week. Four rabbits were completely negative of any antibodies. The rest had dye test titres between 1 and 1:4. They did not show any change in their titres.

Experiment RBLS: Dye Test Antibodies in Splenectomized Rabbits Injected with *Leishmania enriettii* into Tips of Noses

Twenty rabbits, 6–8 weeks old, were splenectomized as before. About three weeks after the last splenectomy, they were bled and *L. enriettii*, obtained from lesions of previously infected guinea-pigs, were injected into the tips of their noses. Dye tests and complement fixation tests were carried out on inactivated sera obtained weekly.

Results.—These are summarized in Table IV. From this group of rabbits, six (RBLS/3, RBLS/8, RBLS/9, RBLS/13, RBLS/16, and RBLS/18) were macroscopically infected with *L. enriettii* which was later confirmed microscopically. One rabbit died at the end of the first week, one rabbit at the end of the sixth week, and two at the end of the seventh week. The rest were kept under observation for another six weeks after the last bleeding at the end of the eighth week. One of the rabbits was completely negative for the dye tests. Once again the titres ranged from 1 to 1:16 with negative complement fixation tests. One of the rabbits which showed a titre in the undiluted serum suddenly increased its titre to 1:16 and remained at this value. This change is not considered as significant.

Experiment GPL: Dye Test Antibodies in Guinea-pigs Infected with *Leishmania enriettii*

Six guinea-pigs which were previously bled by cardiac puncture were injected with *L. enriettii* into the tips of their noses. At the end of the seventh week the guinea-pigs were bled. Eight weeks later they were bled for the last time. Dye tests and complement fixation tests were carried out on the inactivated sera.

Results.—The guinea-pig GPL/1 died three weeks after the second bleeding, and the guinea-pig GPL/4 four weeks after the second bleeding. All the dye tests and the complement fixation tests were negative except in the guinea-pig GPL/1, which showed a dye test titre of 1:256 and complement fixation titres of 1:16 in the pre-infection serum and the post-infection serum. Unfortunately the dye tests and complement fixation tests had not been carried out by this time and hence the brain of the guinea-pig could not be passaged into mice

TABLE III

DYE TEST ANTIBODIES IN RABBITS INJECTED WITH *LEISHMANIA ENRIETTII* INTO TIPS OF NOSES

Rabbit No.	Test	Pre-inoculation Sera	1	2	3	4	5	6	7	8
RBL/1, RBL/2, RBL/3, RBL/5, RBL/6, RBL/7, RBL/9, RBL/10, RBL/11, RBL/12, RBL/20, RBL/22, RBL/23, RBL/24	DT	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4
	CFT	0	0	0	0	0	0	0	0	0
RBL/4	DT	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	
	CFT	0	0	0	0	0	0	0	0	
RBL/8, RBL/16, RBL/17, RBL/18	DT	0	0	0	0	0	0	0	0	0
	CFT	0	0	0	0	0	0	0	0	0
RBL/13	DT	1:4								
	CFT	0								
RBL/14	DT	1:4	1:4	1:4	1:4	0	0	0	0	0
RBL/15	CFT	0	0	0	0	0	0	0	0	0
RBL/19	DT	0	1:4	1:4						
	CFT	0	0	0						
RBL/21	DT	0	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4
	CFT	0	0	0	0	0	0	0	0	0

I = inactivated sera. N = non-inactivated sera. H = haemolysed sera. 1, 2, 3, etc. = weekly sera obtained after infection. DT = dye test. CFT = complement fixation test.

TABLE IV

DYE TEST ANTIBODIES IN SPLENECTOMIZED RABBITS INJECTED WITH *LEISHMANIA ENRIETTII* INTO TIPS OF NOSES

Rabbit No.	Test	Pre-inoculation Sera	1	2	3	4	5	6	7	8
RBLS/1 ..	DT	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4		
	CFT	0	0	0	0	0	0	0		
RBLS/2 .. RBLS/6 .. RBLS/7 .. RBLS/8 .. RBLS/10 .. RBLS/13 .. RBLS/14 .. RBLS/16 .. RBLS/18 .. RBLS/19 ..	DT	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	CFT	0	0	0	0	0	0	0	0	0
RBLS/3 ..	DT	1 : 16	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	CFT	0	0	0	0	0	0	0	0	0
RBLS/4 ..	DT	0	0	0	0	0	0	0	0	0
	CFT	0	0	0	0	0	0	0	0	0
RBLS/5 ..	DT	1	1	1	1	1	1	1 : 16	1 : 16	
	CFT	0	0	0	0	0	0	0	0	
RBLS/9 .. RBLS/11 .. RBLS/12 ..	DT	1	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	CFT	0	0	0	0	0	0	0	0	0
RBLS/15 ..	DT	0	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4	1 : 4
	CFT	0	0	0	0	0	0	0	0	0
RBLS/17 ..	DT	1 : 4								
	CFT	0								
RBLS/20 ..	DT	1 : 4	1 : 4	1 : 4	1 : 4	1 : 16	1 : 16	1 : 16	1 : 16	
	CFT	0	0	0	0	0	0	0	0	

I = inactivated sera. N = non-inactivated sera. H = haemolysed sera. 1, 2, 3, etc. = weekly sera obtained after infection. DT = dye test. CFT = complement fixation test.

for the isolation of *Toxoplasma*. This guinea-pig was, however, considered to harbour a *Toxoplasma* infection or have had some contact with it previously.

Experiment RBCR: Dye Test Antibodies in Rabbits Intraperitoneally Injected with *Crithidia fasciculata*

A culture of *Crithidia fasciculata* was isolated by Professor P. C. C. Garnham from a *Taeniorhynchus* mosquito. This was maintained in the NNN medium with penicillin and streptomycin by Mr. P. E. Nesbitt, who also prepared the inocula used in this experiment. Two rabbits, previously bled, were injected intraperitoneally with 1 ml. of the NNN medium containing the parasites. One week later another injection was given. At the end of the second week from the last inoculation the rabbits were bled. They were again injected with the NNN medium containing the parasites four weeks after the post-inoculation bleeding and once again after a week. At the end of two weeks after the last injection the rabbits were bled for the last time. Dye and complement fixation tests were carried out as before on inactivated sera.

Results.—One of the rabbits was completely negative for the dye test and the complement fixation test antibodies. The other had a dye test titre of 1 : 4 in all three specimens of sera. The complement fixation tests were negative.

Experiment GPA: Dye Test Antibodies in Guinea-pigs Intraperitoneally Injected with *Atoxoplasma sp.*

The *Atoxoplasma* was isolated from sparrows at The Winches Farm, St. Albans, Herts., by Dr. R. Lainson, who supplied me with the spleens of birds infected with the parasite. Six guinea-pigs which were previously bled were injected with ground spleens of four sparrows which were microscopically positive for *Atoxoplasma*. Twelve weeks later they were given another injection of ground spleens containing the parasites. Twenty-four weeks after the first injections the guinea-pigs were bled by cardiac puncture. Dye tests and complement fixation tests were carried out on these sera after inactivation.

Results.—All the dye tests and the complement fixation tests were negative.

Experiment MST: Dye Test Antibodies in Mice Infected with *Trypanosoma cruzi*

Six mice which had been infected about 12 weeks previously with *Trypanosoma cruzi* were passed on to me by Mr. W. Cooper. All of them had been microscopically positive for *T. cruzi* at some time or other. The mice were sacrificed and bled from the heart. Dye tests and complement fixation tests were carried out on these sera.

Results.—All the mice were completely free from dye test and complement fixing antibodies for *Toxoplasma*.

DISCUSSION

Westphal and Knüttgen (1950) obtained negative dye tests in 14 cases of tertian malaria. Similarly, Awad and Lainson (1954b) could not demonstrate dye test antibodies in a rhesus monkey infected with *Plasmodium cynomolgi*. Jacobs (1956) did not obtain definite evidence for the production of dye test antibodies in rats infected with *Plasmodium berghei* or in chickens infected with *Plasmodium gallinaceum*. Cathie (1957) obtained three dye test positives in 76 sera from patients of all ages and with all stages of malaria from Uganda. Since the complement fixation test with the egg antigen was negative, he considered this of no importance. Similarly, Beverley (1957) obtained 35% of 85 cases of malaria in the Forces positive to the dye test at a titre of 1:4 (final dilution) and 2.5% positive in the complement fixation test. He also did not consider this significant as he expected 100% overlap if there was any antigenic relationship. Of the sera of three patients who were examined by Beverley (1957) before and after experimental infection with *Plasmodium vivax*, one serum remained negative and the other two weakly positive before and after infection. Jacobs (1956) could not obtain positive dye tests in a large number of squirrels naturally infected with a species of *Hepatozoon* or in chicks infected with *Eimeria tenella*.

Mühlpfordt (1951) obtained positive dye tests in guinea-pigs, rats, and a hamster infected with *Sarcocystis* experimentally. The guinea-pigs, which were previously negative, developed titres of 1:25 and 1:50 while the rats, which were also negative, developed titres of 1:200 and 1:400. These titres were maintained at the end of eight and twelve weeks respectively. In the hamster the titre was 1:25 at four weeks and 1:200 at seven weeks. In a survey of dye test antibodies in 45 sheep, Mühlpfordt (1951) found 12 sheep with positive dye tests and macroscopic infections of sarcosporidia, 10 sheep with positive dye tests but no macroscopic infections of sarcosporidia, and 23 sheep with negative dye tests and no macroscopic infections. Since macroscopic examination of throat muscles would not show incipient infections and as the rate of infection otherwise was low compared with figures given by other authors, Mühlpfordt (1951) assumed that those showing a dye test titre also harboured *Sarcocystis* infections. Finally, in a guinea-pig harbouring a macroscopic infection of *Sarcocystis*, he obtained a positive dye test titre of 1:50. On this evidence he assumed a cross reaction between *Sarcocystis* and *Toxoplasma*. Awad and Lainson (1954a, b) obtained similar results in sheep infected with sarcosporidia. Of 19 sheep positive for *Sarcocystis*, eight showed dye test titres, while out of six sheep negative for sarcosporidia, three showed dye test titres (Awad and Lainson, 1954b). Six lambs, which were negative in the complement fixation tests using both *Toxoplasma* and *Sarcocystis* antigens, were also negative in the dye test. Therefore Awad and Lainson (1954a, b) assumed the presence of a cross reaction between *Toxoplasma* and *Sarcocystis*. Using the spores of *Sarcocystis tenella* instead of *Toxoplasma*, Awad (1954a) was able to demonstrate dye test antibodies in three human sera showing dye test antibodies by the classical method, in another human serum which was negative by the usual method, and in three guinea-pigs which were experimentally infected with *Toxoplasma*. On the other hand, Moscovici (1953, 1954) could not demonstrate experimentally a serological affinity of a high grade between *Toxoplasma* and *Sarcocystis*. Of 45 sheep, 12 of which were definitely infected with *Sarcocystis tenella*, 22 showed positive reactions with titres between 1:4 and 1:10 (Moscovici, 1954). Guinea-pigs and rabbits, immunized with formalin-killed as well as living sarcosporidia, did not show any dye test antibodies, although they showed complement-fixing antibodies with *Sarcocystis* antigen. Reid and Manning (1956) obtained positive dye tests, mostly to very high titres, in all of 36 sheep, most of which they assumed to be infected with sarcosporidia, but they could not obtain any positive complement-fixation tests. Since these positives were obtained in the presence of accessory factor, they suggested that these were not due to non-specific factors. The isolation of *Toxoplasma* from placentae of sheep made the significance of these results uncertain to them. Cathie (1957) in a dye test survey among 69 sheep observed 54 to give a positive dye test with titres up to 1:16. However, after inactivation 20 sera turned out to be completely negative. On investigating 116 human sera containing both positives and negatives with the dye test using the modification of Awad (1954a), Cathie (1957) found all to give a positive dye test. Many of these sera retained this property of modifying sarcospores even after inactivation by heat. On further investigation Cathie and Cecil (1957) noticed that in 23 human sera, a third of which gave a positive dye test with *Toxoplasma*, all were giving a positive dye test at 1:4 dilution using sarcospores. Ninety-three other sera were also found to contain dye test antibodies using the test of Awad (1954a). On repeating the test with sarcospores and the previous 23 sera after inactivation at 56° C. for 30 minutes, they became negative. On the other hand, Cathie and Cecil (1957) found, in a guinea-pig experimentally immunized with *Sarcocystis* complement-fixing antigen, that inactivation did not completely remove the antibodies detected

with the use of sarcospores but retained them in a much lowered titre of 1:4, although no accessory factor was supplied at all, thus demonstrating conclusively that this substance present in the immunized guinea-pig was not the dye test antibodies as defined by Sabin and Feldman. In 10 sheep selected at random, Cathie and Cecil (1957) found six positive in the complement-fixation test using *Sarcocystis* antigen, and all 10 positive in the dye test with sarcospores. However, on inactivating these sera, this ability to modify fresh sarcospores was lost. On the other hand, tests carried out using old sarcospores and human sera showed that inactivating the sera had no effect in the modification as all sera showed positives whether inactivated or not. Varela, Roch, and Vázquez (1955) studied the sera of eight *Rattus norvegicus* with *Sarcocystis* and found three of them positive with the dye test at a titre of 1:32. Of two *R. norvegicus* with sarcosporidia, *Trichinella* and filaria, one rat showed a dye test positive at 1:32. Jacobs (1956) found three rhesus monkeys infected with *Sarcocystis* dye test negative while Sabin (see Jacobs, 1956) found negative dye tests in some cynomolgus monkeys with *Sarcocystis* infections. Thalhammer (1957) could not demonstrate dye test antibodies in rabbits suffering from sarcosporidiosis. Bakal (see van Thiel, 1958) found *Sarcocystis hirsuta* in hearts of cattle which were dye test positive as well as dye test negative. Awad and Lainson (1954b) reported a human case of sarcosporidiosis which was completely dye test negative. Another case, which was also completely negative, was cited by Beverley, Beattie, and Roseman (1954) and by Beverley (1957). The work of Mühlpfordt (1951), Awad and Lainson (1954a, b), and Reid and Manning (1956) suggests the possibility of cross reactions in infections of *Sarcocystis*. On the other hand, Moscovici (1953, 1954) could not obtain any serological relationship between *Sarcocystis* and *Toxoplasma*. However, Moscovici (1954) also could not obtain positive dye tests in rats, rabbits, and guinea-pigs infected with *Toxoplasma* living as well as killed. The results of Cathie (1957) and Cathie and Cecil (1957) throw some light on this problem. The complete removal of the dye test antibodies found in the sera of sheep after inactivation shows that there are some factors other than the dye test antibodies which are known to be heat resistant. These may be the factors involved in the observations of Mühlpfordt (1951) and Awad and Lainson (1954a, b). The presence of factors capable of modifying sarcospores even in the absence of the accessory factor invalidates the modification of the dye test suggested by Awad (1954a). The results obtained by Reid and Manning (1956) could be due to the same phenomenon observed by Cathie (1957). Human cases of sarcosporidiosis cited by Awad and Lainson (1954b), Beverley *et al.* (1954), and Beverley (1957) give additional support. All subsequent workers unanimously agree that *Sarcocystis* infections do not elaborate dye test antibodies.

Frenkel (1953) found *Besnoitia jellisoni* and *Toxoplasma* to be immunologically distinct. Jacobs (1956) found in some instances of rabbits infected with *B. jellisoni* dye test titres up to 1:16. However, he believed them not to have a cross immunity. Rats harbouring *Encephalitozoon* in the brain were found by Jacobs (1956) to show low dye test titres of 1 or 1:4. He suggested the possibility of cross reactions at low titres between *Toxoplasma* and *Encephalitozoon*. Considering the low titres reported by Jacobs (1956), this could be regarded as insignificant.

Michalzik (1953) obtained 64% dye test positives with a titre of 1:25 or higher in 50 adult women suffering from *Trichomonas vaginalis*. On the ground that this represented a higher percentage of positive dye tests than those reported by other German authors, he assumed that *Trichomonas* infections caused the formation of dye test antibodies. Cathie (1955a) obtained positive dye tests in adult women suffering from *Trichomonas* vaginitis. The sera of four adult men with *Trichomonas* infections had negative dye tests. However, Cathie (1955b) found similar proportions of dye test positives and negatives among comparable groups of women suffering from vaginitis and those free from it. On this ground he concluded that *Trichomonas* infections did not affect the dye test. Reid and Manning (1956) obtained three positive dye tests in seven patients with *Trichomonas* vaginitis. After studying with the dye test 132 women who were microscopically positive for *T. vaginalis* and 110 women microscopically negative for it, Piekarski, Saathoff, and Korte (1957) concluded that *Trichomonas* infections did not produce dye test antibodies. Borgen and Björnstad (1957) found 13 women suffering from severe trichomonad vaginitis for over six months to be completely negative, except one who showed a positive dye test in the undiluted serum. Beverley (1957) found 54% of 46 cases of *Trichomonas* infection in the Sheffield region to give a positive reaction at a titre of 1:4 (final dilution). In rabbits and mice experimentally immunized with living *Trichomonas* cultures, Awad (1954b) obtained high titres of dye test antibodies. The rabbits were initially dye test negative. The control mice used were free of dye test antibodies. On this basis Awad (1954b) concluded that *Trichomonas vaginalis* could produce antibodies detectable by the dye test.

Piekarski *et al.* (1957) repeated the experimental studies of Awad (1954b). Of the 15 rabbits used in the study, 11 showed positive dye test titres between 1:16 and 1:256 in the pre-inoculation sera. After repeated inoculations of *Trichomonas vaginalis* from cultures, no essential differences in titres of the dye test and the complement-fixation test for toxoplasmosis were observed, although the inoculated animals showed strong complement-fixation tests with *Trichomonas* antigen. However, two rabbits which were negative at first became feebly positive with a titre of 1:4 in the dye test. Saathoff (see Piekarski *et al.*, 1957) obtained similar results with mice injected intraperitoneally with *Trichomonas vaginalis*. Borgen and Björnstad (1957) did not find any dye test antibodies in 10 mice inoculated with living trichomonads. Similar experiments in rabbits by Bakal (see van Thiel, 1958) gave identical results in the dye test although an increase in the agglutination titre was observed. The claim of Michalzik (1953) that *Trichomonas vaginalis* infections caused the production of dye test antibodies could be dismissed as his control groups were not similar. This is clearly revealed by the results of the surveys obtained by Cathie (1955a, b), Reid and Manning (1956), Piekarski *et al.* (1957), Borgen and Björnstad (1957), and Beverley (1957). However, the experimental studies of Awad (1954b) cannot be dismissed so lightly. On the other hand the repetition of this work by Piekarski *et al.* (1957), Saathoff (see Piekarski *et al.* (1957), Borgen and Björnstad (1957), and Bakal (see van Thiel, 1958) does not support the observations of Awad (1954b). No explanations could be given as to the results obtained by Awad (1954b).

According to Westphal (see Mühlpfordt, 1951; Thalhammer, 1957), no dye test antibodies were built by trypanosomes. Awad (1954b) obtained titres up to 1:512 in four mice previously infected with *Trypanosoma cruzi* while the control mice were negative. Of 16 albino rats infected with *T. cruzi*, Varela *et al.* (1955) obtained dye test titres of 1:32 in 13 rats. However, Jacobs (1956) could not obtain serological reactions between *Trypanosoma cruzi* and *Toxoplasma* in infected rats. Cathie (1957) observed six out of 15 sera from cases of Chagas' disease to show a positive dye test at low titres which he regarded as insignificant. Bakal (see van Thiel, 1958) could not obtain positive results in animals inoculated with *T. cruzi*, although she was able to establish the infection in the peripheral blood of the inoculated animals. Grönroos and Salminen (1955) could not obtain a correlation between the dye test results and the presence of *Trypanosoma lewisi* in the blood in 108 wild rats, *Rattus norvegicus*. Cathie (1957) found only two of 70 sera from patients suffering from infections of *Trypanosoma gambiense* to give dye test titres of 1:16 or lower. Beverley (1957) found 36% of 17 sera from trypanosome infections from Uganda and Nigeria to give positive dye test at a titre of 1:4 (final dilution) which was not regarded as of importance. Saathoff (see Piekarski, 1958) found that mice and guinea-pigs experimentally infected with *Trypanosoma cruzi* and guinea-pigs infected with *T. evansi* were completely free from dye test antibodies although most of them showed infections with these agents. Bakal (see van Thiel, 1958) could not obtain positive tests in experimental animals infected with *Trypanosoma rhodesiense*. Westphal and Knüttgen (1950) did not observe any dye test antibodies in a case of mucocutaneous leishmaniasis. Thus no cross reactions with the dye test were observed in infections with trypanosomes by Westphal (see Mühlpfordt, 1951; Thalhammer, 1957), Grönroos and Salminen (1955), Cathie (1957), and Beverley (1957). Once again the claim of Awad (1954b) could not be substantiated by the experimental work of Saathoff (see Piekarski, 1958) and Bakal (see van Thiel, 1958), although the work of Varela *et al.* (1955) confirmed Awad's findings. In the present study no dye test antibodies were detected in mice which had been infected with *Trypanosoma cruzi*. In spite of the contradictory claims of Awad (1954b) and Varela *et al.* (1955), the consensus of opinion points to the specificity of the dye test with regard to trypanosome infections.

Varela *et al.* (1955) found six rats positive in the dye test with a titre of 1:32 from 50 rats harbouring intestinal flagellates. Westphal and Knüttgen (1950) could not detect the presence of dye test antibodies in a case of amoebic dysentery.

Considering an increasing titre as the criterion for the production of dye test antibodies, no such phenomenon was observed in the experimental work undertaken in this study although a fluctuation in the titres was observed in rabbits infected with *Eimeria stiedae*. A titre over 1:16 was never recorded. This fluctuation could be attributed to the severe necrosis and fibrosis seen in the livers of these rabbits. Further, repeated weekly withdrawal of blood could be significant in animals suffering severe necrosis of the liver. Hence such a fluctuation should be expected as normal. Practically no variations in the titres were observed in splenectomized rabbits infected with *Eimeria stiedae* and in normal and splenectomized rabbits injected with *Leishmania enriettii*. No positive complement-fixation tests were observed in all four experiments. The observed titre differences between the inactivated and non-activated sera in rabbits both normal and splenec-

tomized may be an exhibition of the phenomenon observed by Cathie (1957) in a lesser degree or may be due to experimental errors. Since this difference seems to be more or less constant, the present author holds the former view. Additional evidence for this view comes from similar observations of Sabin and Feldman (1948), Jettmar (1954), Grönroos (1955), and Cathie and Cecil (1957).

The dye test and complement-fixation test titres shown by the guinea-pig infected with *Leishmania enriettii* is clearly a case of concurrent infection. It is unfortunate that this diagnosis could not be clinched by the isolation of *Toxoplasma* from this animal. The absence of dye test antibodies in animals inoculated with *Atoxoplasma* shows that this parasite is immunologically different from *Toxoplasma*. *Crithidia fasciculata* did not produce any dye test antibodies as expected.

Summing up, so far no protozoan parasite has been conclusively incriminated as producing dye test antibodies which could be detected by the dye test as described by Sabin and Feldman (1948).

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GASTRIC PROTEOLYSIS IN DISEASE

4. PROTEINASE ACTIVITY OF EXTRACTS OF HUMAN GASTRIC ADENOCARCINOMATA

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Resting gastric juice and extracts of uninvaded gastric mucosa from the stomachs of patients with gastric adenocarcinomata digest plasma protein with either two or three maxima below *p*H 5. Egg albumen is digested with one or two maxima below *p*H 5, for the maximum found near *p*H 3.5 with plasma protein is absent. These properties do not differ significantly from those described in normal subjects.

Extracts of gastric adenocarcinomatous tissue from the same patients differ in that plasma protein is digested with only one maximum, at *p*H 3.2 to 3.4, and egg albumen is not digested at all.

The proteinase activity of the carcinomatous tissue probably arises from adenocarcinomatous cells and not from invaded muscle cells or reactive fibrous tissue.

These results could be explained either (*a*) if gastric adenocarcinomatous cells synthesized a quite different proteinase from those of normal gastric mucosa, or (*b*) if they synthesized a proteinase, which resembled those of normal mucosa in possessing the active centre responsible for maximal activity near *p*H 3.5 but in which the centre or centres responsible for maximal activity below *p*H 3, were missing.

Greenstein and Stewart (1942) found that extracts of experimentally induced gastric adenocarcinomata from mice had no proteolytic action upon horse serum and egg albumen at *p*H 1.39 and 5.03, whereas normal mouse gastric mucosa was active at the lower *p*H. Apart from this work there appears to be little known about the proteolytic activity of gastric carcinomata. The experiments that are to be described compare the proteinase activity of adenocarcinomata from the stomachs of eight patients with the activity of adjacent uninvaded gastric mucosa and, in some instances, with the activity of gastric juice and of omental lymph glands containing metastases.

Methods

The methods used to determine proteinase *p*H activity curves have been described previously (Taylor, 1956, 1959a). Stomachs with carcinoma were obtained immediately after surgical removal, and cooled to below 5° C. Gastric mucous membrane remote from the neoplasm was cut away and extracted in 2% (w/v) sodium chloride. Care was taken in preparing the carcinomatous extract to exclude normal mucosal cells by taking a core from the centre of the ulcerated surface through the thickness of the growth into the muscle coat. As much muscle as possible was cut away, but clearly these extracts contain not only adenocarcinomatous cells but also invaded muscle cells and fibrous reactive tissue.

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Results

Uninvaded Gastric Mucosa. — Extracts of uninvaded mucosa from five patients digested plasma protein below *p*H 5 with two maxima at *p*H 2.1–2.3 and *p*H 3.3–3.8 (Fig. 1 and Table I). These maxima are similar to those shown by gastric mucosal extracts of most normal subjects (Taylor, 1956, 1959a). In two patients (Nos. 5 and 6) three maxima occurred, in the *p*H ranges 1.5–2.1, 2.6–2.8, and 3.5–3.6, resembling the maxima shown by a minority (about 15%) of normal subjects and by a majority of patients with peptic ulcer (Taylor, 1959b). The eighth patient gave a curve with two maxima, at *p*H 1.6 and 3.1, which resembled curves obtained from extracts of normal pyloric mucosa (Taylor, 1959c).

The *p*H activity curves for the digestion of egg albumen were determined in four patients (Fig. 2, Table I) and the results again resemble those

TABLE I

PROTEOLYTIC ACTIVITY OF GASTRIC JUICE AND OF EXTRACTS OF UNINVADED MUCOSA, OF ADENOCARCINOMATA, AND OF LYMPH GLANDS CONTAINING METASTASES IN EIGHT PATIENTS WITH CARCINOMA OF THE STOMACH

No. of Patient and Source of Enzyme	Substrate	*p*H 1·4–2·4		*p*H 2·5–4·0			
		Maximum *p*H	Amino-acid N (mg.)	Maximum *p*H	Amino-acid N (mg.)	Maximum *p*H	Amino-acid N (mg.)
1. Gastric juice	Plasma protein	2·3	0·15	—	—	3·3	0·14
Fundic mucosal extract		2·3	0·73	—	—	3·4	0·91
Carcinoma ,,		—	—	—	—	3·3	0·62
Muscle ,,		—	—	—	—	3·1	0·15
2. Fundic mucosal ,,	,, ,,	2·2	0·35	—	—	3·3	0·25
Carcinoma ,,		—	—	—	—	3·4	0·74
Fundic mucosal ,,	Egg albumen	1·8	0·18	—	—	—	—
Carcinoma ,,		Inactive		Inactive		Inactive	
3. Fundic mucosal ,,	Plasma protein	2·2	0·28	—	—	3·4	0·34
Carcinoma ,,		—	—	—	—	3·3	0·52
Fundic mucosal ,,	Egg albumen	1·4	0·17	2·5	0·14	—	—
Carcinoma ,,		Inactive		Inactive		Inactive	
4. Fundic mucosal ,,	Plasma protein	2·1	1·08	—	—	3·7	1·34
Carcinoma ,,		2·2	0·22	—	—	3·5	0·45
Muscle ,,		—	—	—	—	3·2	0·18
5. Gastric juice	,, ,,	1·9	0·42	2·9	0·34	—	—
Fundic mucosal extract		1·5	0·34	2·6	0·35	3·5	0·42
Carcinoma ,,		2·1	0·07	—	—	3·2	0·20
Muscle ,,		—	—	—	—	3·1	0·07
Gastric juice	Egg albumen	1·4	0·18	—	—	—	—
		2·1	0·18				
Fundic mucosal extract		1·5	0·22	—	—	—	—
		2·1	0·17				
Carcinoma extract		1·7	0·08	—	—	—	—
6. Gastric juice	Plasma protein	2·4	0·25	—	—	3·5	0·20
Fundic mucosal extract		2·1	0·59	2·8	0·59	3·6	0·55
Carcinoma ,,		—	—	—	—	3·3	0·32
Carcinoma extract (secondary deposit)		—	—	—	—	3·5	0·08
Carcinoma extract	Egg albumen	Inactive		Inactive		Inactive	
7. Gastric juice	Plasma protein	2·1	0·16	—	—	3·0	0·13
Fundic mucosal extract		2·3	0·43	—	—	3·8	0·42
Carcinoma ,,		—	—	—	—	3·4	0·34
,, ,, (secondary)		Inactive		—	—	3·5	0·14
Fundic mucosal extract	Egg albumen	1·6	0·24	—	—	—	—
Carcinoma ,,		Inactive		Inactive		Inactive	
,, ,, (secondary)		Inactive		Inactive		Inactive	
8. Fundic mucosal extract	Plasma protein	1·6	0·38	—	—	3·1	0·42
Carcinoma ,,		—	—	—	—	3·2	0·28

In the tables and figures the amount of liberated amino-acid N is that of the whole digest, which consisted of 4 ml. buffer, 0·5 ml. or 1 ml. of gastric extract, and 1 ml. of plasma or of 5 % egg albumen.

described in normal subjects, in that the maximum seen with plasma protein at *p*H 3.3 to 3.8 did not occur. Thus a single maximum at *p*H 1.4 to 1.8 or two maxima, the second at *p*H 2.1 to 2.5, were seen.

Carcinomatous Extracts.—In contrast to the uninvaded mucosa, carcinomatous extracts from six patients digested plasma protein with only a single maximum at *p*H 3.2–3.4 (Table I, Fig. 2). The degree of digestion at *p*H 2 was slight, ranging from 0 to 40% of that at the maximum. One patient (No. 5) gave in addition a slight, just significant, maximum at *p*H 2.1 which was thought to arise from contamination with uninvaded mucosal cells. The remaining patient (No. 4) differed from the others in that the carcinoma had invaded, but not ulcerated, the mucosal surface, so that normal cells were included in the extract. The resulting *p*H activity curve did not differ significantly from that of the uninvaded mucosa.

The carcinomatous extracts also differed from those of uninvaded mucosa in that four of the five extracts tested failed completely to attack egg albumen between *p*H 1 and *p*H 5 (Fig. 2 and Table I). One extract (from patient No. 5), which is thought to have contained material from uninvaded mucosal cells, gave a slight maximum at 1.7.

Gastric Juice.—The resting juice of two patients (Nos. 1 and 6) digested plasma protein with two maxima at *p*H 2.3 to 2.4 and *p*H 3.3 to 3.5 and thus resembled the gastric juice of normal subjects. In two other patients two maxima were also shown, but at lower hydrogen ion concentrations, *p*H 1.9 to 2.1 and *p*H 2.9 to 3.0. The gastric juice in these cases behaved like normal pyloric mucosal extracts (Taylor, 1959c).

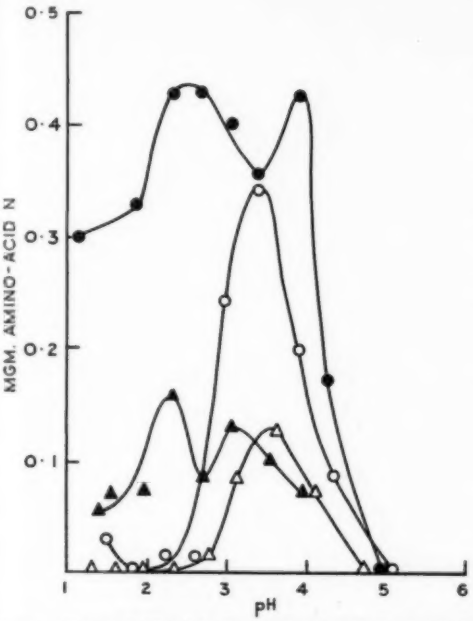

FIG. 1.—Curves of *p*H activity for the digestion of plasma protein by the resting gastric juice (▲), by an extract of uninvaded fundic mucosa (●), by an extract of gastric adenocarcinoma (O), and by an extract from a lymph gland containing secondary deposit (△) from a patient with cancer of the stomach. At 37° C. for three hours.

Lymph Glands with Metastases.—In two patients (Nos. 6 and 7) it was possible to examine omental lymph glands which were almost totally invaded by secondary metastatic adenocarcinomatous cells (Table I, Fig. 1). In both, the extract of the gland digested plasma protein with a single maximum at *p*H 3.5 which closely resembled the maxima seen with the extracts of the primary growths. In one of these patients the lymph gland extract failed to digest egg albumen, again resembling the primary growth. These observations suggest that the proteinase activity of the primary and secondary growths is similar and would support the view that the activity arises from the adenocarcinomatous cells. It has unfortunately not been possible to obtain normal human lymph glands as a control for these observations.

Gastric Muscle.—Since the adenocarcinomatous extracts are almost always contaminated with material derived from invaded smooth muscle cells, their proteinase activity may perhaps arise from these and not from the carcinomatous cells themselves. In three patients, smooth muscle from uninvaded parts of the stomach was extracted in the same manner as the adenocarcinomata and its proteinase activity investigated. The extracts gave slight proteolytic maxima, at *p*H 3.1 and 3.2. In each instance, however, the activity exerted by these purely muscle extracts was only about one-third of that exerted by the carcinomatous extracts, in which the proportion of material derived from smooth muscle must be relatively small. Consequently it is likely that the proteinase activity of the adenocarcinomatous extracts does not arise principally from invaded muscle cells.

Fibrous Tissue. — The adenocarcinomatous extracts also contain material from fibrous reactive tissue, and this, too, might perhaps possess proteinase activity. Fibrous tissue cannot be isolated from the growth itself, but it was possible to obtain from another patient a workable quantity of fibrous tissue from the base of a large benign chronic gastric ulcer. The extract of this fibrous tissue was devoid of proteinase activity at *p*H 1 to 5 when tested upon plasma protein and egg albumen. This observation suggests that reactive fibrous tissue does not contribute to the proteinase activity of the carcinomatous extracts.

Inhibiting Substances.—The absence of a proteolytic maximum near *p*H 2 with adenocarcinomatous extracts might perhaps be caused by an inhibiting substance in the extracts. No inhibition of the activity of normal gastric juice or of crystalline swine pepsin at *p*H 2 was observed when these materials were incubated with carcino-

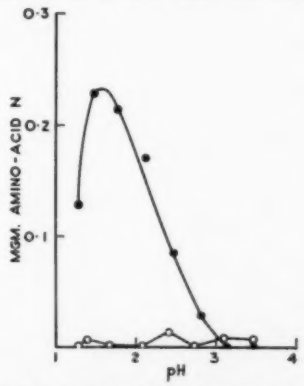

FIG. 2.—Curves of *p*H activity for the digestion of egg albumen by an extract of uninvaded fundic mucosa (●) and by an extract of gastric adenocarcinoma (O) from the same patient as in Fig. 1. At 37° C. for three hours.

matous extract, with dialysed extract, with dialysate from the extract, or with fractions from the extract obtained by ammonium sulphate precipitation.

Discussion

The results reveal one outstanding difference between the proteinase activity of human gastric adenocarcinomata and that of uninvaded gastric mucosa, namely, the absence, with both plasma protein and egg albumen as substrates, of a digestion maximum near *p*H 2. The evidence that the activity of the carcinomata arises from the adenocarcinomatous cells themselves, and not from invaded muscle fibres or reactive fibrous tissue, is very suggestive rather than conclusive, but the absence of the *p*H maximum near *p*H 2 is independent of any uncertainty on this account, for the tumours contained sufficient adenocarcinomatous cells for their extracts to have shown this maximum had they been elaborating the normal enzyme.

If the evidence be accepted that the proteinase responsible for the *p*H maximum at *p*H 3.2 to 3.4 comes from the adenocarcinomatous cells, the question arises as to the relationship of the maximum to that occurring in the same *p*H range with uninvaded mucosa and with gastric juice. The results indicate that the uninvaded mucosal extracts and gastric juice exhibit a wider range of *p*H maximum (*p*H 2.9 to *p*H 3.8) than is exhibited by the carcinomatous extracts (*p*H 3.2–3.4), though this difference might be accounted for by the fact that the uninvaded mucosal extracts and gastric juice contain varying proportions of two proteinases, one from the chief cells of the body of the gastric glands, which in the normal subject exhibits *p*H maxima at *p*H 1.8 to 2.4 and *p*H 3.4 to 3.9, and the other from the neck chief cells and from the pyloric glands, exhibiting maxima at *p*H 1.5 to 1.9 and *p*H 3.0 to 3.4 (Taylor, 1959c). One strong point of resemblance between the enzymes from carcinomatous tissue and those of the uninvaded mucosa is their inability to attack egg albumen when the *p*H is above 3.0.

There is evidence (Taylor, 1959d) that the two proteolytic *p*H maxima exhibited by each of the two normal proteinases of gastric juice and gastric mucosal extracts result from the activity of two active centres on each enzyme. It is possible that the centre responsible for activity around *p*H 3.5 is essentially similar, structurally, in the enzymes from normal tissue and in that from carcinomatous tissue, and that, in the absence of a demonstrable inhibitor, the loss of the maximal activity at *p*H 2 arises from an inability of the adenocarcinoma cell to synthesize an enzyme with two active centres. The alternative explanation would be that the proteinase in gastric carcinomatous extracts is unrelated to those of normal gastric mucosa.

Whether gastric adenocarcinomata arise from normally differentiated gastric cells which undergo neoplastic change, or from undifferentiated cells which undergo neoplastic change, it seems that the absence of a proteolytic maximum near *p*H 2 represents a difference between a neoplasm and the tissue from which it is derived and poses a problem which requires a biochemical answer.

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N-ACETYL-β-GLUCOSAMINIDASE ACTIVITY IN SERUM DURING PREGNANCY

BY

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A spectrophotometric method for the estimation of N-acetyl-β-glucosaminidase in serum has been devised. Sera from normal adult males and females showed similar levels of activity.

The activity in serum rose progressively during pregnancy and fell rapidly after parturition to normal levels. This change resembled closely that which occurs in serum β-glucuronidase.

Placenta showed a moderate and chorion a high level of N-acetyl-β-glucosaminidase. High N-acetyl-β-glucosaminidase activity was demonstrated histochemically in decidual cells.

The functions of N-acetyl-β-glucosaminidase and β-glucuronidase and factors influencing their activity are discussed.

Enzymes which hydrolyse glycosides of N-acetyl-glucosamine occur in a wide variety of biological material. It is probable that their function *in vivo* is to break down oligosaccharides derived from aminopolysaccharides such as hyaluronic acid, which contains β-glucosaminide linkages, or heparin, which contains α-glucosaminide linkages. Synthetic N-acetyl-α- and -β-glucosaminides have been prepared and used as substrates for the demonstration of both types of N-acetylglucosaminidase activity in mammalian tissues (Helferich and Iloff, 1933; Roseman and Dorfman, 1951).

Work on the kinetics and specificity of N-acetyl-β-glucosaminidase has been carried out using partially purified preparations of the enzyme from various sources (Watanabe, 1936; Neuberger and Pitt Rivers, 1939; Pugh, Leaback, and Walker, 1957b). Quantitative data concerning the amount of the enzyme in tissues of rat and mouse have recently been published (Conchie, Findlay, and Levvy, 1959). An extensive survey of the distribution of N-acetyl-β-glucosaminidase in the tissues of the rat has recently been completed in this laboratory and the results are being prepared for publication. High levels of N-acetyl-β-glucosaminidase in epididymis (Conchie *et al.*, 1956) and the secretions of secondary sex organs (Conchie and Mann, 1957) have been described. Hahn (1946) failed to detect any N-acetyl-β-glucosaminidase activity in ox serum.

Following the development of improved methods for the estimation of N-acetyl-β-glucosaminidase (Pugh *et al.*, 1957b) and a study of the factors involved in the estimation of the enzyme in crude tissue preparations (Pugh *et al.*, 1957a), a simple method for the estimation of the activity of N-acetyl-β-glucosaminidase in serum has been devised using *p*-nitrophenyl N-acetyl-β-glucosaminide (Westphal and Schmidt, 1952) as substrate. A preliminary survey has established the range of N-acetyl-β-glucosaminidase activity in normal human sera and an increased activity has been observed in sera from pregnant women.

McDonald and Odell (1947) described changes in the activity of β-glucuronidase in serum during pregnancy. The activity of this enzyme rose from about the third month of pregnancy, reached its highest level in the last few weeks, and fell rapidly to within normal limits after parturition. The activity of β-glucuronidase in cord serum and in homogenates of placenta and endometrial tissue gave no indication of the source of the increased activity in serum during pregnancy. The changes in serum N-acetyl-β-glucosaminidase activity described in this paper are similar to those which have been described in the case of β-glucuronidase. Evidence is presented which indicates that decidual tissue may be the source of the increased level of N-acetyl-β-glucosaminidase in serum and the results are discussed in relation to other work on the functions of the two enzymes.

Methods

Preparation of Substrates.—Phenyl, *p*-nitrophenyl, and α-naphthyl N-acetyl-β-D-glucosaminides were prepared by the method of Leaback and Walker (1957).

Estimation of N-Acetyl-β-glucosaminidase in Serum.—The absorption of *p*-nitrophenyl liberated by N-acetyl-β-

glucosaminidase from *p*-nitrophenyl N-acetyl-β-glucosamide was estimated in alkaline solution at 400 mμ in a "unicam" SP 500 spectrophotometer. The absorption of unhydrolysed substrate at this wavelength is negligible. The following technique was adopted on the basis of the kinetic experiments described below.

Tubes containing substrate (3.6 mM), citrate buffer (0.05 M, *p*H 4.3), and serum (0.1 ml.) in a final volume of 1.0 ml. were incubated at 37° for 30 min. Control tubes in which serum or substrate was omitted were also incubated. Each estimation was carried out in duplicate. At the end of the incubation period the reaction was stopped and the colour of the liberated *p*-nitrophenol developed by the addition of 2 ml. 0.25 M Na_2CO_3. Readings were stable for at least two hours after addition of Na_2CO_3 provided the tubes were protected from light. Photo-catalysed decomposition of substrate can be avoided by using 0.2 M-borate buffer, *p*H 9.8, instead of Na_2CO_3 for stopping the reaction (J. W. Woollen, private communication). After correction for serum and substrate blanks the amount of *p*-nitrophenol liberated by the enzyme was read from a calibration curve constructed with a standard solution of *p*-nitrophenol. One unit of N-acetyl-β-glucosaminidase is defined as the amount of enzyme which will liberate 1 mg. of *p*-nitrophenol under the above conditions. Thus defined, units of N-acetyl-β-glucosaminidase per 100 ml. serum equal μg. *p*-nitrophenol liberated per tube.

Serum was used for all the estimations reported. N-acetyl-β-glucosaminidase in serum is stable for several hours at room temperature and for several days at 0–4°. In a small series of observations the activities of N-acetyl-β-glucosaminidase in serum and heparinized plasma prepared from the same specimen of blood agreed within ±10%.

Estimation of N-Acetyl-β-glucosaminidase in Tissues.—Portions of freshly delivered placentae and membranes were blotted and weighed. After sampling the combined membranes an incision was made at the insertion of the umbilical cord and the amnion was stripped from the placenta and chorion. The tissues were ground with water for 1 min. in a glass homogenizer and diluted to give a concentration of 1% (w/v). N-acetyl-β-glucosaminidase activity was estimated by the method of Pugh *et al.* (1957a, b) using phenyl N-acetyl-β-glucosaminide as substrate. An aliquot of each homogenate was dried at 110° to constant weight.

Localization of N-Acetyl-β-glucosaminidase in Tissue Sections.—The histochemical reaction, which depends on the liberation of α-naphthol by the enzyme from α-naphthyl N-acetyl-β-glucosaminide and its precipitation in the section as insoluble azo-dye by "simultaneous" coupling with diazotized *o*-aminazotoluene, was carried out as described by Pugh and Walker (1958). The fixed tissues were embedded in gelatin (Pearse, 1953) before sectioning.

Results

Kinetic Experiments.—The effect of *p*H on the rate of hydrolysis of *p*-nitrophenyl N-acetyl-β-glucosaminide was studied in 0.05 M citrate buffer over the range *p*H 3.8–5.5. A smooth curve with a single *p*H optimum at 4.3 was obtained. The rate of hydrolysis in 0.05 M citrate buffer, *p*H 4.3, increased with increasing substrate concentration over the range 0.15 to 6 mM. Km for *p*-nitrophenyl N-acetyl-β-glucosaminide, derived by the method of Lineweaver and Burk (1934), was 0.76 mM. At the substrate concentration selected for routine use (3.6 mM) the reaction velocity was nearly maximal. The amount of *p*-nitrophenol liberated from 3.6 mM *p*-nitrophenyl N-acetyl-β-glucosamide in 0.05 M citrate, *p*H 4.3, was shown to be proportional to the amount of serum present (range 0.1–0.4 ml.) and to the duration of the period of incubation employed (range 15–60 min.).

N-Acetyl-β-glucosaminidase Activity in Human Serum.—Table I shows the results of estimations on sera from normal adult males and females (ages 18–50 years). There was no significant difference between the means of the two groups. Table II shows that in five normal subjects only slight variations in activity of serum N-acetyl-β-glucosaminidase occurred during the course of the menstrual cycle. The results of estimations in sera from 120 normal pregnant women are shown in Table III. The subjects were unselected with regard to age and parity. Serial observations on individual subjects were not made. The mean N-acetyl-β-glucosaminidase activity of serum rose throughout pregnancy. The increase was logarithmic, the activity being approximately double in each trimester. Eight days after delivery serum activity had declined to near

TABLE I

N-ACETYL-β-GLUCOSAMINIDASE ACTIVITY IN SERUM OF NORMAL ADULTS

	N-Acetyl-β-glucosaminidase Activity (units/100 ml. serum)		No. of Observations
	Mean ± S.E.	Range	
Males	6·6 ± 1·5	5·1– 9·6	10
Females	6·3 ± 2·0	3·6–11·3	20

TABLE II

VARIATION IN N-ACETYL-β-GLUCOSAMINIDASE ACTIVITY OF SERUM DURING NORMAL MENSTRUAL CYCLE

Day of Cycle	N-Acetyl-β-glucosaminidase Activity* (units/100 ml. serum)				
	A	B	C	D	E
1	6·8	8·7	7·4	6·5	5·3
5	5·6	8·1	6·6	9·1	6·2
14	5·9	7·2	6·9	8·8	7·1
21	5·4	7·3	6·4	7·4	7·5
26	5·3	8·7	7·2	6·1	—

* Subjects A–E were normal women aged 18–30 years.

TABLE III
VARIATION IN N-ACETYL-β-GLUCOSAMINIDASE ACTIVITY OF SERUM DURING NORMAL PREGNANCY

Duration of Pregnancy (weeks)	N-Acetyl-β-glucosaminidase Activity (units/100 ml. serum)		No. of Observations
	Mean ± S.E.	Range	
9–12	13·9 ± 1·1	8·3– 20·5	21
13–16	14·8 ± 4·4	6·8– 23·8	14
17–20	13·5 ± 5·1	6·2– 23·1	9
21–24	23·9 ± 12·3	7·9– 51·6	6
25–28	28·8 ± 13·3	15·2– 54·2	6
29–32	38·6 ± 6·6	31·4– 50·0	6
33–36	42·3 ± 10·4	18·2– 83·7	22
37–40	48·9 ± 14·8	14·7–100·7	21
8 days post-partum	10·3 ± 1·7	8·3– 12·4	8

TABLE IV
N-ACETYL-β-GLUCOSAMINIDASE ACTIVITY IN MATERNAL SERUM DURING LABOUR AND PUERPERIUM AND IN CORD SERUM

	N-Acetyl-β-glucosaminidase Activity (units/100 ml. serum)*							
	A	B	C	D	E	F	G	H
Maternal serum								
In labour ..	42·0	52·4	78·0	38·2	42·4	46·6	42·6	30·3
Puerperium (days):								
1	17·7	26·2	39·2	31·8	—	—	—	—
2	12·1	16·0	15·0	41·2	12·6	19·3	17·6	18·4
4	11·3	18·5	—	31·2	—	—	—	—
8	8·3	13·3	9·2	11·5	8·8	9·3	12·4	9·5
12	9·0	12·7	12·0	13·3	—	—	—	—
Cord serum ..	14·8	29·8	17·8	11·2	16·0	9·1	14·4	10·9

* Serial specimens were obtained from subjects A–H at the indicated times.

the normal level. This rapid fall was studied in more detail in a small group of patients from whom sera specimens were obtained in labour and during the puerperium (Table IV). With one exception, the decline in serum N-acetyl-β-glucosaminidase activity was most rapid during the first 48 hours after delivery. In subject D the fall did not take place till after the fourth day of the puerperium. Changes in activity between the eighth and twelfth days were only slight. In this group of cases N-acetyl-β-glucosaminidase activity in serum from cord blood was also estimated. The results show that the activity in foetal serum was in each case considerably lower than that of the corresponding maternal specimen obtained during labour. The mean activity of foetal sera (15.5 units/100 ml.) was, however, higher than that of normal adults (Table I). In Fig. 1 the mean serum N-acetyl-β-glucosaminidase activity (data from Table III) is plotted against duration of pregnancy. Activities have been expressed as percentages of the activity at term in order that they may be compared with similar data provided by McDonald and Odell (1947) for the activity of β-glucuronidase in serum during pregnancy. The extent and course of the changes in the activity of the two enzymes are very similar.

Activity of N-Acetyl-β-glucosaminidase in Tissues.—N-acetyl-β-glucosaminidase activities of human placenta and foetal membranes were investigated since these tissues are possible sources of the high serum activity found in association with pregnancy. The results are shown in Table V. Activity was present in both placenta and membranes and most of the activity in the latter was associated with the outer chorionic layer. The activities are referred to a dry weight basis since these tissues contain relatively large amounts of water. No data for N-acetyl-β-glucosaminidase activity of other human tissues are available, but in comparison with normal rat tissues the activity of placenta was moderate, similar to that of liver or spleen, while the activity of chorion

TABLE V
N-ACETYL-β-GLUCOSAMINIDASE ACTIVITY OF PLACENTA AND FOETAL MEMBRANES

Tissue*	N-Acetyl-β-glucosaminidase Activity (μg. phenol liberated/mg. dry weight tissue)	Dry Weight of Tissue (% wet weight)
Placenta (8) ..	68·1	17·2
Combined membranes (8) ..	130·2	12·9
Amnion (6)	17·6	12·5
Chorion (6) ..	193·5	12·5

* Results are the mean of the number of observations shown in parenthesis.

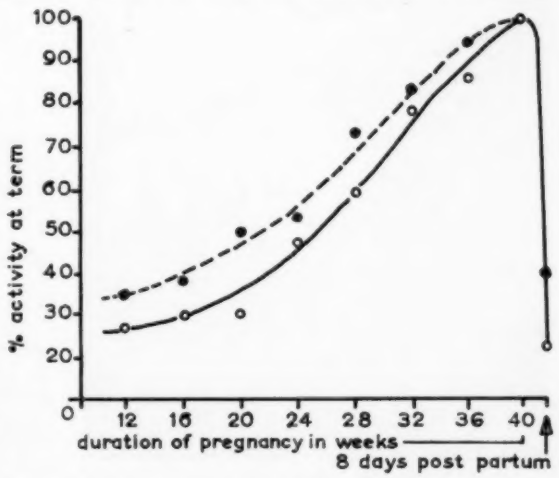
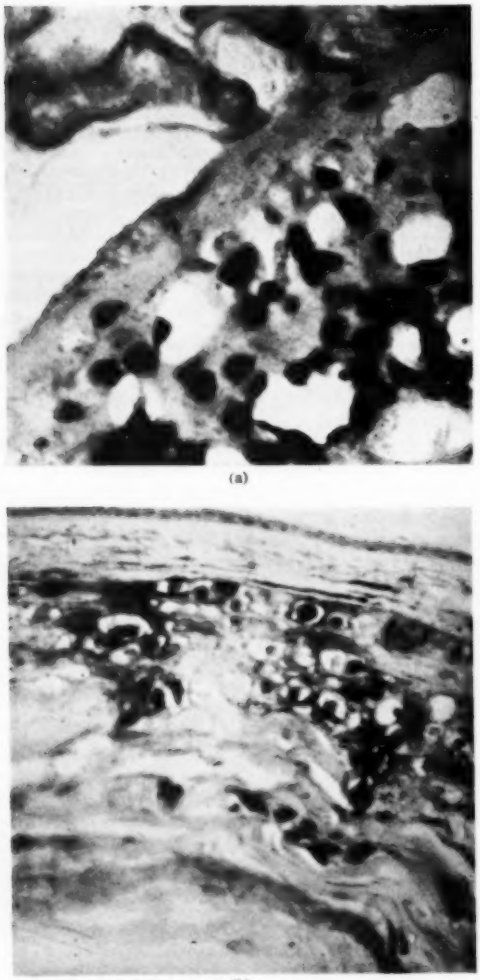


FIG. 1.—Variation in mean N-acetyl-β-glucosaminidase (O——O) and β-glucuronidase (● – – –●) activity of serum during normal pregnancy. Activities expressed as % activity at term. Data for β-glucuronidase from McDonald and Odell (1947).

was high, similar to that of epididymis, the most active of the normal rat tissues (Conchie *et al.*, 1956).

Localization of N-Acetyl-β-glucosaminidase in Tissues.—The histochemical localization of N-acetyl-β-glucosaminidase in formalin-fixed gelatin-embedded placenta and membranes is shown in Fig. 2a and b. Decidual cells in the basal and chorionic plates of the placenta and on the maternal surface of the chorion show an intense reaction. Amnion, chorionic connective tissue, and placental villi are much less active.



(a)

(b)

FIG. 2.—Localization of N-acetyl-β-glucosaminidase in (a) basal plate of placenta and (b) foetal membranes. Decidual cells in both sections contain a heavy deposit of azo-dye indicating high enzyme activity. ×150.

Distribution of N-Acetyl-β-glucosaminidase in Whole Blood.—Experiments were carried out to test the possibility that the N-acetyl-β-glucosaminidase activity of serum might be affected by haemolysis or by leakage of enzyme from cells during the separation of serum. The experiments were technically unsatisfactory, but are described briefly because they show that neither of the above factors is likely to have influenced the results obtained. Plasma and a frozen-thawed suspension of saline-washed cells were prepared from heparinized blood. The standard assay method had to be modified because of the high blanks given by whole blood or cell suspension. Trichloroacetic acid was used to stop the enzyme reaction and the reaction mixture as deproteinized by centrifuging. *p*-Nitrophenol in an aliquot of the supernatant was estimated after addition of Na_2CO_3. The recovery of *p*-nitrophenol in the supernatant was only 75–85% complete, the remainder being adsorbed on to the precipitated protein. The results obtained, however, showed that in normal subjects about 90% of the N-acetyl-β-glucosaminidase in whole blood was in the plasma and only slight activity was found in the blood cell suspension.

Discussion

The interpretation of these observations is necessarily speculative. It is usually assumed that an increase in the activity of an enzyme in serum reflects an increased level of production by tissues. The significance of the moderate N-acetyl-β-glucosaminidase activity of placenta and the high level in the foetal membranes is difficult to assess because no estimations on other tissues from normal or pregnant human subjects have yet been carried out. However, the intense activity of N-acetyl-β-glucosaminidase demonstrated histochemically in decidual cells and the rapid post-partum decline in the level of the enzyme in maternal serum make it probable that the changes in activity of serum during pregnancy are associated with changes in the production of the enzyme by the endometrium.

The parallel changes in activity of N-acetyl-β-glucosaminidase and β-glucuronidase in serum during pregnancy suggest that they may be related. Evidence that the activity of both enzymes is under hormonal control has been provided by the work of Fishman and Fishman (1944) and Kerr, Campbell, and Levvy (1949) on β-glucuronidase, and by Conchie and Findlay (1959), who showed that low levels of N-acetyl-β-glucosaminidase in ovariectomized mouse uterus were restored to normal by

administration of oestrone. The changes in activity of these enzymes in serum during pregnancy may therefore reflect a hormonally conditioned alteration in tissue activity. The validity of the hypothesis of McDonald and Odell (1947) that the increase in β-glucuronidase activity during pregnancy occurred adaptively in response to the need for increased formation of steroid glucuronides is doubtful in the light of recent work, which has shown that β-glucuronidase is not involved in the synthesis of glucuronide detoxication products (Dutton and Storey, 1954).

In another context, a link between N-acetyl-β-glucosaminidase and β-glucuronidase was provided by the work of Linker, Meyer, and Weissmann (1955) on the breakdown of hyaluronic acid. Hyaluronic acid is composed of chains of alternating, glycosidically linked residues of glucuronic acid and N-acetylglucosamine. Hyaluronidase, an "endo-glucosaminidase," forms oligosaccharides by the random hydrolysis of β-glucosaminide linkages within the chain; these are further degraded to monosaccharides by the successive actions of β-glucuronidase and N-acetyl-β-glucosaminidase which remove terminal non-reducing sugar residues. Changes in the activity of β-glucuronidase and N-acetyl-β-glucosaminidase during pregnancy may be associated with alterations in the metabolism of mucopolysaccharides and of the connective tissue cells from which such ground substances are derived. For example, changes in the extracellular polysaccharide of the symphysis pubis and of the foetal membranes were demonstrated by the work of Frieden and Hisaw (1953) and Harkness and Harkness (1956).

The results described indicate the need for further observations on the N-acetyl-β-glucosaminidase activity in serum and tissues in different physiological and pathological states. Preliminary figures suggest that the activity of the enzyme in rabbit serum increases during pregnancy, and an attempt will be made to correlate this with changes in tissue levels and histochemical localization.

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DETERMINATION OF MAGNESIUM BY THE TITAN YELLOW AND AMMONIUM PHOSPHATE METHODS

BY

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The ammonium phosphate and Titan yellow methods for determining magnesium have been investigated, and improvements to both techniques are presented. The Titan yellow method is shown to be subject to interference which makes it unsuitable for the analysis of blood cells, food, and faecal materials, and may also affect its reliability with pathological samples of serum and urine. The relative insusceptibility to interference of the ammonium phosphate method makes it more reliable than the Titan yellow method with pathological specimens, and slight modifications permit the use of the same basic procedure with blood, urine, and solutions of food or faecal ash.

The wide variety of methods which have been employed for the determination of magnesium in serum serve to illustrate the fact that no technique is completely satisfactory. Methods which may be carried out directly on diluted serum, in some cases after deproteinization, include combination with dyes such as Titan yellow and Eriochrome black T, titration with ethylene diamine tetra-acetic acid, and flame photometry. Other methods involve the initial separation of magnesium, usually as the insoluble magnesium ammonium phosphate, followed by colorimetric estimation of the phosphate in this precipitate. Despite the variety of these techniques, very few have been developed to permit the determination of magnesium in clinical materials other than serum.

Detailed investigations of magnesium metabolism require a method which is suitable not only for the determination of serum and plasma magnesium, but which may also be applied to the estimation of magnesium in urine, blood cells, and acidic solutions of food or faecal ash. These considerations led to the detailed study of methods based on two well-established principles which are widely employed for the estimation of magnesium in serum.

Titan Yellow

This method depends on combination between Titan yellow and magnesium hydroxide to produce an orange-coloured lake, but unfortunately the resulting increase in colour is comparatively small compared with the colour of the free dye. The procedure developed by Neill and Neely (1956) is to be preferred to the techniques described by Orange and Rhein (1951) and Andreasen (1957), as it produces a higher ratio of colour intensity/blank, but the method may be improved by introducing the following modifications:

(1) Replacing gum ghatti by 0.1% polyvinyl alcohol, which increases the range of magnesium concentration over which the calibration curve is linear.

(2) Treating the standard magnesium solutions with deproteinizing reagents in the same manner as the test samples, thus allowing for the effect of these reagents on the intensity of colour developed.

(3) Increasing the accuracy of the final stage by measuring the colour produced in cells of 2 cm. light path with a spectrophotometer at 550 mμ.

The calibration curve must be constructed with each series of determinations as the intensity of colour produced varies slightly from day to day and decreases progressively with increasing age of the Titan yellow solution. The method is very sensitive to any trace of contamination, but good reproducibility can be maintained by routine cleaning of the tubes used for colour development with chromic acid.

This method is normally satisfactory for the analysis of serum and urine, but allowance must be made for the calcium content of test samples as described by Neill and Neely. Although some Titan yellow techniques are apparently unaffected by the presence of calcium (Baron and Bell, 1957), with the above procedure the addition of 10 mg./100 ml. calcium to a solution containing 2 mg./100 ml. magnesium raises the apparent magnesium content by 7%.

However, interference studies have shown the method to be affected by many substances besides magnesium, and this restricts its use with materials other than serum and urine. The depression of colour development produced by iron, which was reported by Orange and Rhein, has been confirmed in this laboratory, and a similar effect has been observed with citrate; the apparent magnesium content of serum is lowered by 15% in the presence of 50 mg./100 ml. citric acid. Gillam (1941) and Otto and Otto (1941) have demonstrated the potential interference of phosphate, ammonium, and many other cations, but the concentration of these ions in serum and urine is usually insufficient to produce inaccuracy. Recently Barker, Elkinton, and Clark (1959) have reported inaccuracy caused by the presence of gluconate and mercurial diuretics.

Magnesium Ammonium Phosphate

Several micro-modifications of the classical ammonium phosphate precipitation technique for determining magnesium after the prior removal of calcium have been developed. The method for estimating serum magnesium described by Simonsen, Westover, and Wertman (1947) is suitable with the following modifications:

(1) The calcium oxalate precipitate is washed once with 2 ml. of 2% ammonium hydroxide and the washings added to the original supernatant, thus preventing the loss of 4% magnesium by adsorption on the calcium containing precipitate.

(2) The magnesium ammonium phosphate precipitate is allowed to form overnight; although two hours normally suffice, complete precipitation may take longer with some pathological specimens.

As the preparation and washing of the magnesium containing precipitate rather than the precision of the final colour measurement is the stage limiting the ultimate accuracy of the method, the molybdivanadate method for estimating the phosphate content of this precipitate (Simonsen, Wertman, Westover, and Mehl, 1946) is preferred to the molybdate I and II methods of Fiske and Subbarow (1925); the greater stability of the colour produced with the former method more than outweighs the greater sensitivity of the latter. The calibration curve constructed from theoretically equivalent phosphate solutions is exactly reproducible, but it is desirable to include both a blank and a standard magnesium-calcium-containing solution with each series of determinations in order to check the absolute accuracy of the method. Optimum accuracy is obtained with samples containing 20–160 μg. magnesium, and it is undesirable to use more dilute specimens as difficulty in manipulating the small amount of precipitate may lead to low results.

The same basic procedure can be employed for the determination of magnesium in specimens other than serum. After laking with water the magnesium content of blood cells is estimated in the same way, the results obtained being in agreement with those from ashed cells. The analysis of urine and food or faecal ash requires preliminary adjustment of the *p*H for precipitation of calcium oxalate. This is achieved by adding 20% sodium acetate solution dropwise to the acidified sample until the neutral point of methyl orange is reached, when the estimation is completed as for serum.

Although the method is rather slow and laborious, routine use over a period of one year has shown it to produce satisfactory results with an average reproducibility of 2 to 3%.

Apart from calcium, which is initially removed as the oxalate and may thus be determined readily by titration with permanganate, the only substance known to interfere with this method in the concentrations at which it may occur in clinical material is iron. Erroneously high results are obtained if more than 500 μg. of Fe^{3+} is present in the sample assayed, but this amount is unlikely to be encountered except in samples containing more than 0.5 ml. of ashed blood cells.

Comparison

Serum and urine from a small series of normal individuals have been analysed simultaneously by both methods. The results with serum indicate a systematic difference, the ammonium phosphate technique consistently yielding results 5–7% higher than those given by the Titan yellow method. However, with urine the differences between the methods appear to be random.

Although comparisons have not been made with a wide variety of pathological specimens, the Titan yellow method has been found to under-estimate seriously the magnesium content of urine from patients receiving Shohl's citric acid/sodium citrate solution (Albright, Consolazio, Coombs, Sulkowitch, and Talbott, 1940) and the method is therefore unreliable in such cases.

Conclusion

Direct methods of analysis are potentially more rapid in use and more sensitive to small changes in concentration than methods which involve an initial separation of the substance to be determined, but frequently they are also more susceptible than the latter to undesirable interference. Experience with

the two methods considered above supports this generalization.

The ammonium phosphate separation procedure may be used to determine magnesium in serum, urine, blood cells, and food or faecal material with only slight modification of the same basic procedure. Moreover, the method is virtually free from interference by other substances occurring in clinical specimens. Undoubtedly the chief disadvantage of this method lies in the two-day period which is required to complete a series of analyses.

The relative speed and sensitivity of the Titan yellow technique make it an attractive procedure for determining magnesium, but unfortunately the method is not highly specific and interference is produced by many substances, both organic and inorganic in nature, which may be encountered in clinical materials. The method is normally suitable for use with serum and urine, but this susceptibility to interference makes it unsatisfactory for the analysis of blood cells, food, and faeces, and may also lead to erroneous results with some pathological samples of serum and urine.

I wish to thank Miss M. Nicholson for assistance.

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TECHNICAL METHODS

A Spectrophotometric Method of Estimating Blood Oxygen Using the Unicam SP 600

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Accurate estimations of percentage oxygen saturation and oxygen capacity are frequently needed in research and routine investigation of patients suffering from diseases of the heart and lungs. Blood gas analysis for this purpose is time consuming and requires the services of a highly skilled technician. Further, the methods need to be in constant use if reliable results are to be obtained. Accurate results require duplicate or triplicate estimations.

The increasing use of cardiac catheterization in diagnosing congenital heart disease has created a need for a simple and reliable method for rapid estimations. A number of authors have described spectrophotometric methods designed to suit different types of instrument (Nahas, 1951; Wade, Bishop, Cumming, and Donald, 1953; Stainsby, Fales, and Lilienthal, 1955). In the present paper we describe a modification of the method of Holling, MacDonald, O'Halloran, and Venner (1955) suitable for the "unicam" SP 600. Individual determinations can be made in less than two minutes and the method is suitable for use in the dark at catheterization. It thus provides a simple and reliable technique for estimations of oxygen saturation on blood samples during the course of catheterization.

Methods

Blood gas analysis was performed by the method of Van Slyke and Neill (1924). Duplicate estimations of the oxygen content were made on each sample. Where a number of samples was taken the oxygen capacity was measured on one sample. A cuvette similar to that described by Holling *et al.* was constructed out of heavy gauge brass sheeting and ¼ in. perspex sheet. Two cells were constructed by making suitable holes in an "alkathene" sheet 5/1,000 in. thick (Fig. 1). The cell was mounted rigidly beneath a carriage similar to that used for the standard liquid cells. Filling and drainage tubes connected each cell to the upper surface of the carriage, the filling tube ending in a Luer socket and the drainage tube in a convenient bevel (Fig. 2). When the carriage was placed in position on the instrument the cells coincided with the light beam of the absorptiometer. Blood for estimation by blood-gas analysis and by spectrophotometry was taken into 10 ml. syringes suitably greased containing a small quantity of liquid heparin. Mercury was introduced into the syringe, which was then capped and the blood and heparin mixed by agitating the mercury. The samples were kept cold until estimations were made. Blood gas analysis by the Van Slyke method was made by using 2 ml. of blood for each estimation. All estimations were made in duplicate, and, in those in which agree-

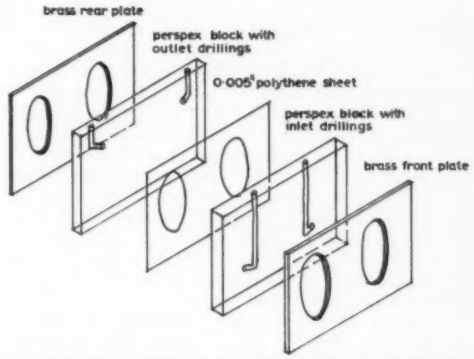

FIG. 1.—Exploded view to show construction of cell. The depth of the chamber in which the blood is contained is determined by the thickness of the polythene sheet. The fixing screws have been omitted from the drawing.

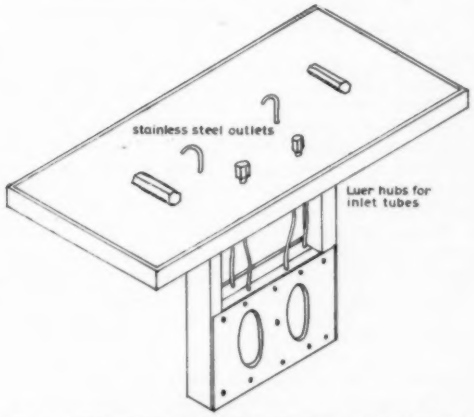

FIG. 2.—The chamber attached to the carriage. Only a few of the fixing screws are shown.

ment was not within 3%, a third estimation was made. For the spectrophotometric estimation a 1 ml. syringe was first rinsed with a freshly prepared saturated solution of white heparin and the dead space filled with this solution. A short length of alkathene tubing (about 3 mm.) was passed over the nozzle of the Luer syringe containing the blood and the 1 ml. syringe fitted on to the other end of the alkathene tube. Approximately 1 ml. of blood was then passed into the small syringe and a small quantity of mercury added. The saponin solution and the blood were then mixed by agitation. Haemolysis was usually complete in 15 to 20 seconds and the haemolysed blood solution was introduced into the cuvette through the lower socket in the top of the carriage. It was found most convenient to remove the carriage from the spectrophotometer and place it horizontally against a piece of white paper to ensure that no large bubbles were left in the cuvette. The cuvette not filled with blood was filled with 0.4% ammonia solution as a reference cell. The optical density was then measured at 480 mμ and 506 mμ and the percentage oxygen saturation calculated by the method described by Holling.

$$\%\text{saturation} = \frac{x \text{ unknown sample} - \text{reduced haemoglobin}}{x \text{ oxyhaemoglobin} - x \text{ reduced haemoglobin}} \times 100$$

where x is the ratio $\frac{\text{optical density at 480 m}\mu}{\text{optical density at 506 m}\mu}$

The optical densities of reduced haemoglobin and oxyhaemoglobin need only be calculated for a single sample of blood as this ratio is independent of the haemoglobin concentration. It follows that in the equation for calculating the percentage saturation only the ratio of the unknown sample is a variable and the remaining terms are constants. It is therefore possible to simplify the calculation by constructing a nomogram relating the ratio for the unknown sample of optical density at 480 mμ to the optical density at 506 mμ to its percentage oxygen saturation. This is shown in Fig. 3.

After use the instrument is cleaned by rinsing through with 0.4% ammonia solution which may be left in the cuvettes. Should it be found necessary to dismantle the cuvette for cleaning, the perspex surfaces should be wiped with lens paper moistened with ethanol. The presence of one or two minute bubbles in the blood contained by the cuvette does not disturb the estimations. Small particles of mercury should be removed by rapid flushing with ammonia solution. Blood clots render the estimations unreliable and may necessitate dismantling and cleaning. They should not occur if the saponin and blood are adequately mixed.

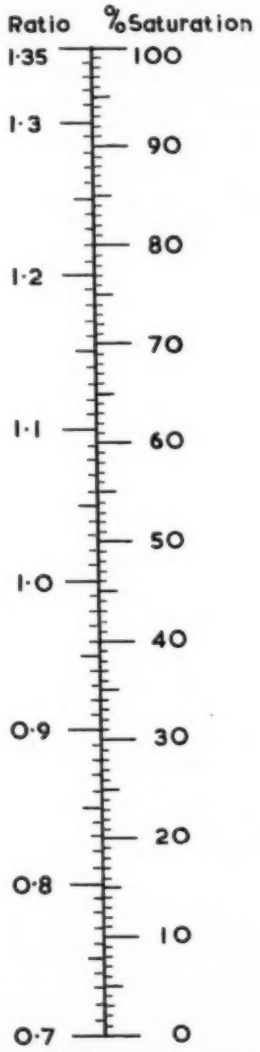

FIG. 3.—Nomogram relating percentage saturation to x, the ratio of the optical density of the unknown sample at 480 mμ and 506 mμ. This ratio is first calculated with a slide rule and the percentage saturation read from the nomogram; e.g., a ratio of 1.09 indicates an oxygen saturation of 60%.

Results

The absorption curves for reduced haemoglobin and oxyhaemoglobin were first determined in the cuvette. Using two different samples of blood no significant difference from the curves shown by Holling *et al.* was found.

Simultaneous estimations of percentage oxygen saturation were made on 50 samples of blood taken during cardiac catheterization using the Van Slyke and spectrophotometric methods. In three cases the results were discarded because the samples were too small to permit duplicate Van Slyke measurements. The results are shown in Fig. 4. The majority of the results agreed closely.

Those in which a divergence was found from the Van Slyke estimations had been unsatisfactory because only a small quantity of blood was obtained so that the calculation of percentage saturation had been made on the oxygen capacity of another sample taken during the same catheterization. The percentage difference between the results obtained by Van Slyke and the SP 600 are shown in Table I. In 31 cases the difference was 2% or less and in only four was it greater than 5%.

TABLE I

Percentage Difference between Estimates	Number of Estimations
0	10
1	6
2	15
3	5
4	4
5	3
6	0
7	1
8	1
9	2
Total	47

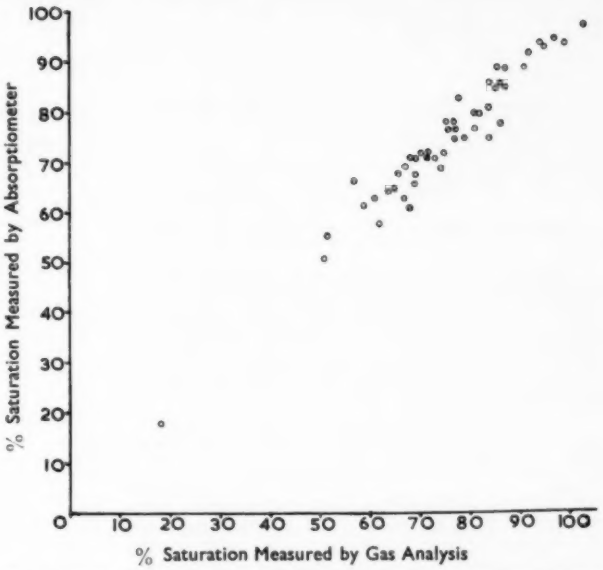

FIG. 4.—Graph relating the estimations of percentage saturation by absorptiometry and by gas analysis.

The injection of coloured dyes (Evans blue, Coomassie blue, and Fox green) in the course of cardiac catheterization is becoming an increasingly common practice. It seemed desirable therefore to investigate the effects of concentrations of these dyes upon the estimate of percentage saturation with the SP 600. Coomassie blue was added to whole blood in varying concentrations up to one part of 4% solution in 250 parts of blood. This is equivalent to the injection of 20 ml. of 4% solution into an average human subject. Although there was a marked increase in optical density of the blood the determination of percentage saturation was unaffected. Similar results were obtained with Evans blue and Fox green.

Conclusion

The method described here provides a rapid and convenient means of determining percentage oxygen saturation using a reliable spectrophotometer which is available in most laboratories. This has been shown to be unaffected by the addition of coloured dyes to the blood and is suitable for immediate use during cardiac catheterization.

In subsequent comparisons of the two techniques 29 samples were tested. Of these, 16 gave identical results in both methods, seven showed a 1% difference, four a 2%, and two gave a 3% difference.

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Improved Egg Candler for Virus Work

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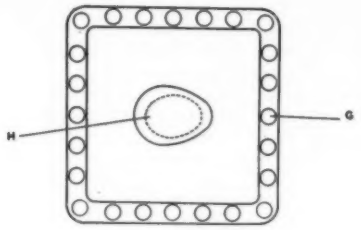
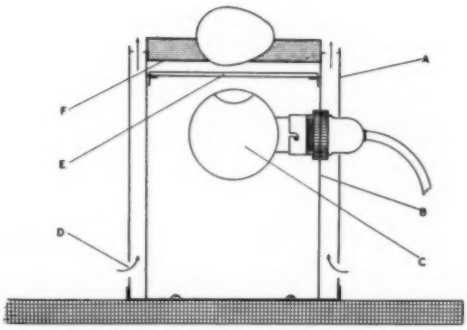
Working with fertile eggs it is necessary before inoculation to make certain that the embryo is living, and to locate the positions of the air sac and embryo. The usual equipment for this purpose consists of an airtight box containing a 100-watt electric light bulb with an oval opening over which the egg is placed (Beveridge and Burnet, 1946).

Such a simple device becomes very hot, particularly after prolonged use, and this is rather undesirable when manipulations are carried out under direct vision. For example, it is convenient to complete the dropping of the chorioallantoic membrane under direct vision when using the perforator described by Nadeje, Tamm, and Overman (1955). The following design was found useful in practice.

The apparatus consists of a double-walled container, the outer wall (A) being perforated at the top and bottom by a ring of holes (D and G). During use the inner skin (B) becomes hot, heating the air between it and the outer layer. The hot air is convected upwards and escapes through the upper ring of holes, while cool air is drawn in at the bottom. In this way convection is used as a means of cooling and light traps are unnecessary. As the source of light a Lister type of ophthalmic lamp was chosen since this gives maximal light and minimal heat. The bulb has a choice of three apertures; for purposes of the candler the largest size was found most suitable. The lamp should be set approximately 6 cm. from the egg. During the manipulations an egg may break occasionally, and when this happens and the fluid contents drop on the lamp, it bursts. For this reason a glass screen is interposed between the egg and the lamp (E).





The metal top of the candler has an oval hole cut into it (H) which is approximately 5 cm. long and 3.5 cm. wide. A sheet of nylon foam ½ in. thick is stuck to the top of the candler (F) and the edges bevelled around the hole. This cushion moulds itself to the egg, giving a light-proof seal.

The box is mounted on a base board approximately 12 in. square to give stability during the use of the apparatus.

This apparatus is obtainable from Messrs. Matburn Ltd., 20–24, Emerald Street, London, W.C.1.

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Effect of Crystallized Trypsin of Increased Enzymic Activity in Trypsin Tube Tests *

N. A. F. YOUNG

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(RECEIVED FOR PUBLICATION OCTOBER 20, 1959)

Armour Laboratories are now producing crystallized trypsin of increased enzymic activity and their most recent product has a potency of 2,500 units/mg.

During the development of the "concurrent trypsin method" the trypsin used had a potency of only 1,000 units/mg., and the trypsin concentration of 0.48%, recommended as being optimal in the serum-cell mixture, therefore corresponded to an enzymic activity of 480,000 units%. As stressed in the original publication, the "concurrent method" is sensitive only within a fairly narrow range of trypsin concentration, but it was not made clear that this critical concentration is in fact dependent upon the potency of the enzyme used, since what is important is that the enzymic activity in the incubated mixture must be maintained between 300,000 and 500,000 units%. The strength of the stock trypsin solution must therefore be varied according to the potency of the crysallized trypsin reagent (see Table).

	Crystallized Trypsin (Armour Laboratories)		
	1,000 units	2,500 units	3,000 units
Stock trypsin solution (g.%)	3·00	1·25	1·00

It should be carefully noted that if the 2,500 units/mg. crystallized trypsin is used at the concentration advised in the original paper the test becomes insensitive and weak antibodies will be missed. Furthermore with a stronger antibody (such as would be used for Rh grouping), although positive reactions will be obtained, the agglutinations will be much weaker and less clear-cut than they should be.

The trypsin-saline method has now been used for the routine Rh grouping of almost 8,000 cases with excellent results.

It offers certain very definite advantages, namely, (1) simplicity and speed of performance; (2) considerable economy of grouping serum; (3) more sensitive, reliable, and clear-cut results than albumin methods. Rouleaux formation is no problem and the obvious distinction between the positive and negative tubes considerably lessens the chances of "false positive" readings being recorded by less experienced workers; (4) grouping sera can be used suitably diluted (usually 1/8 to 1/10) so that D^u samples either do not react or give weak microscopic agglutination.

All negatively reacting samples are screened by the Coombs technique and possible D^u samples further investigated.

* Addendum to "Studies on concurrent sensitization and trypsinization with the development of a simple trypsin tube test for routine Rhesus grouping and as a screening test for incomplete antibodies" (*J. clin. Path.*, 1957, **10**, 236) and "Direct compatibility testing" (*J. clin. Path.*, 1958, **11**, 311).

The Use of a Dyeline Photocopier in a Hospital Laboratory

R. L. McCORRY

From the Tyrone County Hospital Laboratory, Omagh

(RECEIVED FOR PUBLICATION MARCH 17, 1960)

New systems for recording results in hospital laboratories have been described by Maxwell (1957) and by Brown, Gillespie, McGowan, and Raper (1959). In their systems the form received with the specimen is a two-part combined request-report form with carbon paper interleaved. In the blank report section, the laboratory stamps a proforma. So far as possible results are recorded directly on the form; the original is returned to the ward and the carbon duplicate is retained in the laboratory.

Photocopying is an alternative to carbon paper for making duplicate copies. Of the methods available (*Office Magazine*, 1958) we have found the dyeline system simple, rapid, and economical. This system has been used mainly by architects for copying plans; in recent years small continuous-feed machines have been produced for office use. The copy paper is coated with a diazo compound which is bleached by light waves in the blue region and which darkens when treated with a suitable coupling solution. The copy paper may be safely handled in full daylight. Documents printed on translucent but not necessarily transparent paper may be used as negatives to produce positive copies.

In this laboratory single-part request-report forms are used which are essentially similar in layout to those described by Brown *et al.* (1959). The forms are printed on paper (Wiggins Teape penetrator) specially designed for use with dyeline systems; it has the appearance of a medium-weight typing paper. Proformas are printed with embossed metal plates in an "addressograph-multigraph" 500 machine (Maxwell, 1957; Brown *et al.*, 1959). Where possible results are recorded directly upon the form. The completed form is copied in an Ilford "azoflex 105" dyeline photocopier; the original is returned to the ward. In this continuous-feed machine, forms can be copied at the rate of four per minute; a single copy takes 20–25 seconds. Copy paper costs less than ½d., the form itself a little more than ½d. The machine saves a considerable amount of the time of the laboratory secretary; it can be and is used by any member of the laboratory staff.

My thanks are due to the medical, nursing, and laboratory staff of this hospital for their co-operation; to the Northern Ireland Hospitals Authority for a grant for equipment, and to Mr. Thompson and Mr. Burge of the O and M department for advice.

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BOOK REVIEWS

Atlas der Phasenkontrasthämatologie. By Hansjurgen Rind. (Pp. 392; 1,094 figures. £6 15s.) Berlin: Akademie-Verlag. 1958.

Hämatologischer Atlas. By Horst Stobbe. (Pp. 304; 236 figures. £5 19s.) Berlin: Akademie-Verlag. 1959.

During much of the history of clinical pathology the literature has been greatly indebted to German workers. Most senior pathologists were brought up on their textbooks, which were always characterized by minute attention to detail and exhaustive if not always critically analysed references. Excellence of colour printing and of photographic reproduction was also commendable.

Much of this leadership was lost during the political upheavals of the middle years of this century; and meanwhile the vast expansion of American and British work has deviated attention from German contributions. It is a pleasure therefore to welcome these two complementary studies, which fully adhere to the high standards of traditional German work, both pictorially and academically.

Phase-contrast microscopy has perhaps been most revealing in the study of the cells of the blood and bone marrow, as from these sources cells can most easily be obtained in a fresh state, without undesirable manipulation such as is involved in trypsin dispersal. Cytoplasmic control in particular has for the first time been fully revealed, and there is little doubt that fresh classifications, particularly in leukaemia, await workers in this field.

The atlas of phase-contrast haematology edited by Hansjurgen Rind is a massive work, including an elaborate exposition of technical methods. There are more than a thousand illustrations, copiously annotated, together with a reasonably complete list of references. Due credit is paid to Bessis, one of the earliest pioneers.

Horst Stobbe contributes a section to this work, and his own haematological atlas is complementary since it deals mainly with stained preparations, but includes phase-contrast correlations; moreover, Rind's atlas gives a particular emphasis to the haematology of childhood. There are detailed commentaries on the excellent illustrations, and a full discussion of technical procedures.

R. J. V. PULVERTAFT.

The Thyroid Gland. [*Brit. med. Bull.*] (Pp. 86. 20s.) London: British Council. May, 1960.

The high standards associated with the *British Medical Bulletin* have been worthily upheld by the latest number on the thyroid gland. In a short but masterly introduction Sir Charles Harington not only places in perspective the rapid advances made during the past decade but indicates some of the more important unsolved problems.

The formation of thyroid auto-antibodies, possibly the most dramatic of recent discoveries, is authoritatively reviewed by I. M. Roitt and D. Doniach, who discuss the value of such tests in the diagnosis of Hashimoto's disease and certain thyroid disorders.

The genetic aspects of thyroid disease are considered by F. D. Kitchen and W. Howel Evans and by E. M. McGirr, who describes a fascinating study in which sporadic goitrous cretinism was manifested in no fewer than 13 different members of a single family. W. R. Trotter discusses the association of deafness with thyroid dysfunction and concludes that in cases of Pendred's syndrome there is strong evidence for a common genetic abnormality responsible for both defects whereas endemic deaf-mutism is largely the result of environmental rather than genetic factors.

Recent work on the biosynthesis of thyroid hormones is capably summarized by R. Pitt-Rivers, while the significance of protein binding in relation to their transport is discussed by J. R. Tata. A convincing explanation of the differences in the physiological activities of thyroxine and tri-iodothyronine is given.

G. S. Boyd and M. F. Oliver have reviewed the effects of thyroid hormones and related compounds on plasma cholesterol levels, and have concluded that certain analogues may be of therapeutic value in coronary heart disease. Limitations of space do not permit more than mere mention of the other contributions, which deal with types of thyroid growth, genesis of nodular goitre, the diagnostic use of radio-iodine, naturally occurring goitrogens, placental transport, and the interrelationships of the thyroid with the pituitary, the central nervous system, and the hypothalamus.

The Scientific Editor, Dr. N. B. Myant, is to be heartily congratulated on the excellence of this Bulletin, which will prove invaluable to all interested in the thyroid whatever their specialty might be.

J. H. WILKINSON.

Diagnostic Radioisotopes. By Charles A. Owen, Jr. (Pp. xix+425; 70 figures, 49 tables. £6 6s.). Oxford: Blackwell. 1959.

A number of competent books on this and related subjects have been published in recent years, and it is against these that this book must be judged. All of them face a common problem: that of achieving a satisfactory balance between, on the one hand, discussion of the purely physical problems of radioactivity measurement, and the theoretical considerations upon which the isotopic techniques are based, and, on the other hand, descriptions of the methods currently in use and interpretation of results. Some succeed better than others in the balance achieved; this book fails completely.

The chapters on counting techniques are largely useless and are in no way improved by the many illustrations of boxes of electronic equipment which have no difficulty in preserving their anonymity behind their front panels. The theoretical bases of some of the problems are poorly explained, others, e.g., those involving multicompartment systems, are not discussed at all.

The allotment of space to methods and interpretations bears little relation to their importance; for instance, 10 pages are devoted to the problem of B_{12} absorption, but only 13 to the whole of the cardiovascular system, electrolyte and fluid spaces, and protein metabolism. Indeed, these latter subjects cannot be said to have been properly presented at all.

There are extensive but largely uncritical reviews of the literature relating to medical isotopes, and 64 out of the 381 pages of text are devoted to pointless tables including logarithm tables and that ubiquitous du Bois surface area nomogram.

You may have all this for six guineas.

C. J. HAYTER.

The Anti-Globulin (Coombs) Test in Laboratory Practice. By I. Dunsford and Jean Grant. (Pp. xiii+120; 11 tables, 9 figures. 12s. 6d.). Edinburgh and London: Oliver and Boyd. 1959.

This book is admirably written by two leading authorities on the subject of the antiglobulin reaction or Coombs test. The test has not only proved of great importance in the advancement of knowledge in various biological sciences with particular reference to blood groups, but it is widely used in hospital blood transfusion practice for the prediction and diagnosis of haemolytic disease of the newborn and also to enhance the safety of many other blood transfusion procedures. The authors explain the fundamentals of the test from all angles in a style which is both lucid and informative. They include comprehensive references to the world literature and make suggestions for further reading which may help in relation to individual problems. The work, which is delightfully produced and of modest price, is recommended strongly. It should be on the bookshelves of bacteriologists, haematologists, blood transfusion workers, forensic and veterinary scientists, and last, but not least, those medical students and practitioners interested in that field.

E. K. BLACKBURN.

Forensic Medicine. By A. Keith Mant. (Pp. vii+262; 114 figures. 42s.) London: Lloyd-Luke. 1959.

This is a further addition to the already numerous small textbooks of forensic medicine. It may be classified as one of the "horrific" class from the point of view of some of the illustrations, for similar photographs have caused comment by members of the legal profession in the past, but it is difficult to see why juries should be spared them if they are of value. For police officers, the approach is inclined to be too medical, and it is elementary for pathologists and incomplete for general practitioners, one of whose main interests must be the problems of "alcoholic intoxication" which, somewhat surprisingly, are omitted.

The production is very good and the illustrations well printed, but it seems a pity that many of the individuals could be recognized. It does not seem to be an "essential" for the bookshelf of clinical pathologists, medico-legally interested or otherwise.

F. CAMPS.

A Textbook of Bacteriology, 8th ed. revised and enlarged. By R. W. Fairbrother. (Pp. vii+502; 7 plates, 32 tables. 25s.) London: Heinemann. 1959.

Fairbrother's well-known textbook is now in its eighth edition and retains the characters of reliability and conciseness which have made it so valuable to medical students. It now includes brief reference to many of the newly described viruses as well as to the newer antibiotics and other recent developments, but these have not managed to oust a good many references to ideas or to methods that are now superseded.

R. E. O. WILLIAMS.

The Biochemical Response to Injury. Report of a symposium organized by C.I.O.M.S., Vienna, 1958. (Pp. xi+467; illustrated. 57s. 6d.) Oxford: Blackwell Scientific Publications. 1960.

This is a report of a symposium organized by C.I.O.M.S. following the fourth International Congress of Biochemistry in Vienna in September, 1958. The Chairman was very appropriately Dr. D. P. Cuthbertson, whose pioneer work on the metabolic response to trauma 30 years ago has stood the test of time, and on whose early observations has been built much of the more recent work. It is sometimes doubtful how useful is the publication of symposia, which may have been of the greatest value to the participants, but are of less lasting general interest; there is no doubt that this publication is very well justified: it deals with a subject of growing importance, from both the experimental and clinical points of view. "Shock" is still a condition which it is impossible to define accurately, but the more we know of the metabolic changes which occur after trauma the nearer we are to understanding it, and the more rational will be the treatment.

The participants include many of the leading workers in this field from all over the world, and the contributions give a very stimulating approach to many problems. This is a book which should be widely available, and should be referred to frequently, not only by pathologists, but even more by surgeons.

E. N. ALLOTT.

J. clin. Path. (1960), 13, 369.

RADIOACTIVE ISOTOPES IN MEDICINE: A REVIEW

BY

C. J. HAYTER

From the Isotope Laboratory, St. Thomas's Hospital, London

The following account is very selective: no attempt is made to deal exhaustively with radioisotope applications. In particular, practically no mention is made of treatment using, e.g., ^{131}I, ^{198}Au, or ^{90}Yt, or of the literature dealing with the localizing of tumours, much of which is unprofitable. Circulatory dynamics, e.g., cardiac output, are ignored, as are most biochemical investigations which have no direct clinical bearing as yet. Radiation dosimetry for individual tracer quantities and the organs most affected are to be found in the quoted references; recommendations and data for maximum doses are to be found in "Code of Practice for the Protection of Persons Working with Ionizing Radiations in National Health Service Hospitals" (1957). London, H.M.S.O.

Logical division into subject groups is almost impossible, and topics are discussed under the headings of counting techniques, haematology, chemical pathology, thyroid metabolism, and general considerations.

The Radiochemical Centre, Amersham, is the source of most radioactive materials, many of them processed, e.g., ^{58}Co-labelled vitamin B_{12}, and publishes stock and price lists which are readily available.

Counting Techniques

Geiger Muller Tubes.—These are both cheap and versatile. A large range of sizes, shapes, and characteristics is obtainable commercially, and some special modifications are made for specific purposes. "End window" counters with a specially thin window of material, which minimizes absorption of weak β rays, have been in use for some years. Much of the early work with ^{35}S and ^{14}C was done with these counters, and provided the amount of activity present in a sample (which is dried on to a disc or planchette of standard size) is of a reasonable order, nothing better is required. Sensitivity is very low, since apart from self-absorption considerable absorption occurs even with very thin mica windows (less than 1 mg./cm.^{2}), and this is the greater with decreasing energy of emission. Tritium (^3H) cannot be counted for this reason. The technique has been reviewed (Libby, 1947).

Improved counting efficiency (three- or four-fold) is obtained by placing the sample within the counter. Self-absorption problems remain, but absorption due to intervening air and window is no longer a factor. Geometrical conditions are also improved. The correct pressure and quench gas filling must be maintained: a suitable apparatus is some form of gas flow proportional counter (Simpson, 1947; Taylor, 1953) where gas is allowed to flow through the chamber at atmospheric pressure to maintain the correct gas conditions in spite of leakage. Certain modifications, some with automatic sample changing devices, are available commercially. Expense is increased by the high amplification needed, but the apparatus is simple to use, and, for many purposes, satisfactory.

A useful modification of Geiger Muller tubes is the liquid sample β counter (Veall, 1948), in which a thin-walled tube is surrounded by an outer glass wall which contains the liquid sample to be measured. The geometrical conditions for counting are good, and give the most efficient means of counting energetic β emitters such as ^{42}K and ^{32}P. They are particularly useful for counting samples containing mixed isotopes in multiple isotope techniques.

Although Geiger Muller tubes are inefficient counters of γ rays, this does not matter provided there is enough activity present. Geiger Muller tubes can therefore be used for a number of purposes where they replace the more costly crystal scintillation counters. Rings of Geiger Muller tubes, made to accommodate, e.g., bottles containing 24-hour urine saves or cartons containing whole faecal specimens (Veall and Vetter, 1952; Veall, Lowe, and Whyard, 1955b), are most useful in the laboratory.

Scintillation Phosphors.—The standard γ-ray counting equipment in most laboratories are the well crystal and directional counter. The former consists of a block of phosphor with a central well into which a sample of a few millilitres contained in a glass tube is placed. Photons emitted from the phosphor are converted to electrical signals and amplified in a photomultiplier valve (Anger, 1951). The latter is of the same basic construction, but the crystal is surrounded by any desired shape of lead shielding, so that the counter "sees" a roughly cone-shaped volume of radioactive material (usually the tissues of a patient). The directional counter is used in many kinds of body surface counting, especially when activity in a particular organ is to be measured.

The most sensitive crystals are of thallium-activated sodium iodide, but their cost is very high. For precise differentiation of narrowly separated energy peaks in mixtures of isotopes this type of crystal is the best

and may be necessary in some multiple tracer techniques (Kahn and Lyon, 1953), but for most purposes the plastic phosphors now available are good enough. These are produced in many sizes and shapes and are very much cheaper than sodium iodide crystals. Availability and cheapness make possible more sensitive counters than Geiger Muller tube rings for large volume work, together with photomultipliers which have also been enlarged to give increased areas of light-sensitive cathode.

But the problem of very weak β emitters remains. Tritium, in particular, cannot be counted using these methods, and the very small quantities of ^{14}C used, by reason of its long biological and radiological half-life, in human tracer studies cannot be measured accurately by the counting methods already mentioned. Other techniques have therefore been developed.

Gas Counting.—The sample is converted to a gas by combustion and the gas then led into a Geiger Muller tube or ionization chamber (Banks, Crawhall, and Smyth, 1956 ; Miller, 1947 ; Brownell and Lockhart, 1952).

The technique is laborious, requires considerable skill in operation, and has a complicated vacuum system for transferring samples. The sample is destroyed in preparation, and rather complicated chemical preparation may be necessary. On the other hand, counting efficiency is almost 100%, due to elimination of absorption losses. As an ordinary laboratory tool, however, the disadvantages are too great.

Liquid Scintillation Counting.—The sample is dissolved in the same liquid as soluble plastic phosphor. Once again absorption losses are very small, the radioactive atoms being intimately mixed with the phosphor. Efficiency depends then on optics and the photomultiplier. This is an oversimplification, and there are numerous snags. Unless the sample is soluble in the organic solvent which dissolves the phosphor, its volume is limited to the amount which can be got into solution using, e.g., a water-alcohol-solvent system. Any pigment in the sample diminishes light transmission, and proteins tend to be precipitated, producing opalescence which again interferes with light transmission. (Increased solubility of various substances can be attained by using some quaternary amines, e.g., hyamine (Radin, 1958).) The molecular structures of the compounds to be counted may themselves produce a quenching effect (Kerr, Hayes, and Ott, 1957). A further difficulty is the "noise," adding to background, of the dark current produced by the photomultipliers. In the United States commercial equipment is available using photomultipliers "in coincidence." No signal is accepted by the counting circuit unless it arises simultaneously in the two photomultipliers, i.e., the signal arises due to the advent of a photon in the phosphor and not to a random impulse in either photomultiplier. In addition, the whole apparatus is refrigerated since the dark current increases with temperature. The resulting equipment is efficient, with very low backgrounds, for tritiated water and other ^3H-compounds, but is enormously costly and bulky.*

Recent developments with single photomultiplier systems have shown that similar efficiency is obtainable by using an improved photomultiplier of small light-cathode area, and by paying close attention to optics. Equipment is much less bulky, does not require refrigeration, and is only a fraction of the cost. Efficiency is about equal, and quantities of about 0.1–0.2 millimicrocurie of ^3H per sample can be counted.

In addition to the small volume scintillation counters described above, large volume detectors have been developed. These are mainly used for whole body counting, large plastic phosphors being arranged in batteries, or a large annular tank, filled with liquid scintillator and capable of accommodating, e.g., a human adult, is used (Hayes, 1956 ; Bird and Burch, 1958). Although whole body counters using Geiger Muller tubes in suitable geometrical form (Fowler, 1957 ; Veall, Fisher, Browne, and Bradley, 1955a) are quite adequate in some metabolic turnover studies, their sensitivity is low, and spectrographic separation of γ rays is not possible. Large volume scintillation counters, however, are capable of measuring, e.g., the body content of naturally occurring ^{40}K.

Ionization Chambers.—For routine counting in the laboratory ion chambers are not widely used, although, since the chamber can be large, and the gas filling is not critical, accurate measurement of very low specific activity gas samples, e.g., of ^3H and ^{14}C, can be counted. However, extremely high quality small current measurements are necessary, using, e.g., a vibrating reed electrometer, and the apparatus is not cheap. Nevertheless, the ion chamber is widely used for radiation monitoring, and most radiotherapy laboratories use them. If available, an ion chamber is extremely useful for measuring quantities in the order of tens or hundreds of microcuries, and thus tracer doses of various isotopes can be measured and checked simply in a way which is not possible with other routine laboratory counting devices (Veall, 1952).

Haematology

Blood Volume and Red Cell Survival.—The use of ^{51}Cr as a label for red cells (Gray and Sterling, 1950) has largely superseded methods involving other radioactive labels, such as ^{32}P. The labelling process, using the patient's own blood, is simpler, incubation is unnecessary, and the number of washings reduced (Mollison and Veall, 1955). The half-life of ^{51}Cr, 27 days, is more nearly that of the red cells, and the rate of loss of the label from the cells is less rapid and more

* For an exhaustive discussion of liquid scintillation counting problems see *Liquid Scintillation Counting* (1958). C. G. Bull, Jr., and F. N. Hayes (Editors). Pergamon Press, London.

predictable (Hughes Jones and Mollison, 1956). ^{51}Cr from old cells is not reincorporated into new cells. The mechanism of labelling is thought to depend on the fact that hexavalent chromium can pass through the red cell membrane, after which it is reduced to the trivalent form and bound on to cell protein: on breakdown of the cell the trivalent chromium thus released cannot pass through the membranes of new cells.

Of other isotopes used in red cell labelling, ^{32}P (Hevesy, Köster, Sørensen, Warburg, and Zerahn, 1944; Reeve and Veall, 1949; Chaplin, 1954) has many comparative disadvantages, but can be used when simultaneous volume or short-term survival measurements are made, since it can be differentiated by physical means from ^{51}Cr. It also has the advantage that it can be measured by Geiger Muller counting and crystal scintillators are not necessary. ^{42}K can be used in the same way as ^{32}P (Yalow and Berson, 1951); it has the advantage of a very short half-life (12.4 hours) when repeated red cell volume measurements over a short period are needed.

In spite of the simplicity of ^{51}Cr labelling, the technique requires care, and a standard procedure should be used. There is evidence that the subsequent elution rate from injected labelled cells is dependent on the method of preparation, and, in addition, chromate in high concentration is toxic to cells (Hughes Jones and Mollison, 1956).

Blood Volume.—Injected ^{51}Cr-labelled cells are presumed to be distributed throughout the vascular space in a short time. Ideally, several samples are taken over a period of, e.g., 30 minutes, and the slope obtained extrapolated back to zero time and theoretical instantaneous mixing (Sterling and Gray, 1950). Loss of label by elution is quite small over the period, and the slope is usually quite flat after an initial mixing period of about five minutes in the normal, so that commonly only one sample is taken at 10 minutes and extrapolation ignored. Equilibration may be delayed in some abnormal circulatory states, e.g., shock and cardiac failure (Noble and Gregersen, 1946). In these circumstances simplified procedures may not give valid results.

Counting is done on whole blood using a well type scintillation counter, and the red cell volume, and, indirectly, the whole blood volume, obtained using the venous haematocrit value, to which a small correction is usually applied for trapped plasma (Chaplin and Mollison, 1952). From simultaneous measurement of red cell volume and plasma volume, using a different indicator for each, it has been shown (Chaplin, Mollison, and Vetter, 1953) that the venous haematocrit ratio is not representative of the whole body R.B.C./plasma ratio. (Capillaries contain comparatively more plasma than do larger vessels (Everett, Simmons, and Lasher, 1956; Gibson, Seligman, Peacock, Aub, Fine, and Evans, 1946). In general, whatever the venous haematocrit, there is a relationship between it and the whole body R.B.C./plasma ratio, so that multiplication by 0.91 gives a valid correction. It is stated that the exception is in the patient with anaemia and splenomegaly, in which case the whole body R.B.C./plasma ratio may be greater than the venous haematocrit ratio (Verel, 1954; Rothschild, Bauman, Yalow, and Berson, 1954).

Recent work, simultaneously using ^{51}Cr-labelled cells for red cell volume and ^{132}I-H.S.A. (human serum albumin) for plasma, has demonstrated that when the blood volume is rapidly increased by using "plasma expanders," such as dextran and albumin, the body R.B.C./plasma ratio may vary widely from the venous haematocrit. Indeed, it appears that in some circumstances parts of the vascular volume become inaccessible to the label, and this inaccessibility may vary in different ways for plasma and red cells, the absolute red cell volume appearing to shrink while the plasma volume expands (Hayter and Clapham, 1960). It may therefore be necessary, in some abnormal circulatory states, to have reservations about the applicability of the "whole body haematocrit" correction factor.

The haematologist is usually specifically interested in the red cell volume, but direct measurement of plasma volume may be needed, for instance, when values for total plasma iron are required (see Iron Metabolism), or when simultaneous measurements of red cell and plasma volumes are needed to give more precise measurement of total blood volume. ^{131}I-H.S.A. has the advantage of relative simplicity in the measurement of the indicator concentration in the plasma, and ^{131}I or ^{132}I-labelled human serum albumin provides a suitable indicator for plasma volume measurement. Compared with dyes, ^{132}I-H.S.A. combines this with repeatability, the short half-life of the ^{132}I (2.3 hours) preventing the build-up of high concentration of indicator from estimation to estimation. Iodinated human serum albumin can be conveniently prepared in the laboratory, although it can be obtained ready made from the Radiochemical Centre, Amersham. Laboratory preparation is mandatory for ^{132}I-H.S.A., of course, because of its short half-life (Veall, Pearson, and Hanley, 1955c; Cook, Eakins,

and Veall, 1956). About 10% of a given dose of human serum albumin disappears from the circulation in an hour (Pritchard, Moir, MacIntyre, and Inkley, 1955) so that when mixing time is expected to be prolonged it is even more important to extrapolate through several readings.

Some interest has been shown in the possibilities of rapid and simple methods of measuring blood volume in emergency, e.g., in acute blood loss due to injury or post-operatively. Quick methods designed to be used, perhaps by the house surgeon, have been devised. It is doubtful whether assessment of blood volume diminution by this means offers any advantage over clinical observation. If acute cardiac failure (with which sudden blood loss may be confused) is liable to give anomalous results, there is a distinct possibility that a transfusion might be given where it is absolutely contraindicated.

Values quoted for blood volumes vary over a wide range; they are usually expressed as millilitres per kilogram of body weight. Since the distribution of blood through fatty and lean tissues is not equal, and since fat/lean ratio is so variable in humans, weight as an index is imprecise. It has been shown that when a more precise index is used (in this case "lean body mass") differences in red cell volume of quite small degree can be demonstrated (Muldowney, 1957). Further reference to this is made in the section on body fluids and electrolytes.

Red Cell Survival.—The Ashby technique of differential agglutination (Ashby, 1919), although capable of giving good information on cell survival, is difficult to perform, is very laborious if enough cells are counted to give reasonable accuracy, and, apart from demanding a proper compatibility relationship between the donor and recipient bloods, it also assumes that the donated cells will survive in the same manner as the recipient's. In addition, there is evidence that normal cells will survive less well than the patient's in acquired haemolytic anaemia (Hughes Jones and Mollison, 1956). In spite of apparent blood group compatibility, the survival is sometimes curtailed in an unexpected way, presumably due to unrecognized antibody (Loutit, Mollison, and Young, 1943; Jandl and Greenberg, 1957). Primarily, however, it is the technical difficulties which have prevented its wide use clinically. Apart from the relative simplicity of ^{51}Cr labelling and counting, cell survival study using this technique has the advantage that the patient's own cells are reinjected, so that with certain reservations their survival should be exactly similar to that of the red cell population. In addition, tests of survival of possibly incompatible donor red cells can be performed (Mollison and Cutbush, 1955, 1958), and data can be obtained on the useful life of stored blood under different conditions (Chaplin and Mollison, 1953).

When a random sample of red cells is labelled and reinjected into a red cell pool, provided that the sample consists of a normal distribution of cell age, their survival could follow two main patterns. If the cells are assumed to have a finite life span, a constant proportion will disappear in each unit of time, until, if the percentage surviving were plotted against time, zero would be reached at a time equivalent to the cell life. In fact such a linear plot would show some tailing off towards the end, which would depend on the variance of cell lives about the mean, but extrapolation of the linear part of the curve to zero would give the mean cell life. It is this pattern which has been mainly demonstrated in normals using the Ashby technique, and which gives the accepted mean cell life figure of 100 to 120 days. On the other hand, it can be assumed that the cells are potentially immortal, and that they are destroyed by some random haemolytic process, in which case, if a constant quantity were destroyed in each unit of time, the numbers of labelled cells would diminish exponentially. In this case, the mean cell life would be that time at which 37% of the cells remain. In practice, the results obtained are something of a combination of the two processes, the former behaviour being most marked when there is an intrinsic cell defect, and the latter when there is a marked haemolytic process. The expected behaviour will be modified by a number of factors: if the age distribution of the cells in the labelled sample is abnormal (and this will be so in the case of impaired cell survival); if the cells are not uniformly susceptible to an extrinsic destructive agent; and if the rate of production of cells is fluctuating during the time of survival measurement the shape of the curve will be influenced. Theoretical consideration of the above factors is given by Dornhorst (1951).

When a sample of *in vitro* ^{51}Cr-labelled cells is reinjected into a patient, there is a rapid early loss (over two to three days) of about 10% of the label followed by a slower, predictable rate of elution (Ebaugh, Emerson, and Ross, 1953; Mollison and Veall, 1955). Corrections for these losses can be made (Hughes Jones and Mollison, 1956). The type of correction (tabulated factors for which are given in the previous reference) will depend on the type of curve. An empirical correction factor,

allowing for the initial rapid loss, and relating the actual survival time to the conventional Ashby survival slope, is applied to the " linear intrinsic defect " type of slope, and simple correction for slow elution to the exponential " haemolytic " type.

Interpretation presents some difficulties. The corrections for elution do not produce the linear slope expected for normal cell survival, and although mean cell life might be obtained from the early part of the curve, the distortion produced by the initial rapid loss of ^{51}Cr makes this difficult, especially in interpreting the curves of only slightly abnormal cell survival. Mean cell life as an expression of cell survival is therefore not satisfactory. Expression in terms of half-life of the ^{51}Cr-tagged cells is also objectionable on the grounds, first, that when the regression rate is slow, considerable extrapolation may be necessary, and, second, that use of the half-life concept depends on a strictly exponential decline which can only be true when random destruction is greatly in excess of loss due to age (Dornhorst, 1951).

It has been shown (Mollison and Veall, 1955) that in normal subjects survival curves plotted on semi-logarithmic paper (ignoring the " early loss " period) deviate from linearity little, up to 50 days after injection of labelled cells since the loss of ^{51}Cr is very slow; when the slope is steep, it is likely to be due to an increase in the rate of random destruction, and so the slope will even more nearly approximate an exponential. Using this empirical method the time at which 50% of the labelled cells are still in the circulation has been used as an expression of cell survival, and this is variously given as 25 to 32 days in the normal. The disadvantage in practice is that the length of time for which the survival must be followed in order to express results in this form is sometimes too prolonged (up to 30 days) when the survival is not grossly impaired, although in cases of special interest it may be desirable, since, as in measurement of any constant rate of change, the longer the rate of ^{51}Cr loss is measured the greater will be the accuracy of the measurement.

It has been shown, however (Wetherley-Mein, Epstein, Foster, and Grimes, 1958; Jones, Wetherley-Mein, Ingram, and Langmead, 1960), that if the values for ^{51}Cr activity are plotted on semilogarithmic paper for seven to 10 days, starting 48 hours after the injection of labelled cells (thus excluding the period of rapid early loss), and the best-fitting straight line drawn through the points, the percentage loss per day of ^{51}Cr can easily be calculated. By this method rates of loss greater than about 5% are abnormal. Although a figure of less than 5% per day over such a short period does not necessarily mean that the survival is normal it does exclude any gross degree of haemolysis. As mentioned above, smaller degrees of impairment of survival would necessitate a longer period of study. The advantage of the technique is that it can readily be applied in routine clinical " work-outs " and can be combined with ^{59}Fe studies, i.e., plasma iron clearance, ^{59}Fe turnover and utilization, and surface counting (Wetherley-Mein, Hutt, Langmead, and Hill, 1956; Wetherley-Mein *et al.*, 1958). The method is also useful in before-and-after studies such as assessing the effects of treatment.

An ingenious method for testing the compatibility, which may be doubtful, of donor blood has been demonstrated. A small quantity of the donor cells is labelled with ^{51}Cr and a control sample of the possible recipient's with ^{32}P. The short-term survival of the donor's blood is then compared with that of the control in the recipient, and the presence of serologically unidentified antibody, if present, is detected (Mollison and Cutbush, 1955, 1958).

Surface Counting. — Directional scintillation counting over the liver and spleen when labelled cells are injected shows a slow and small rise in the normal presumably due to destruction of cells and/or accumulation of ^{51}Cr by these organs (Hughes Jones and Szur, 1957). In haemolytic anaemia, the quantity of ^{51}Cr in the spleen, and sometimes the liver, may be very large, and in some cases the removal of the spleen has been followed by good haematological remission (Hughes Jones and Szur, 1957; Jandl and Greenberg, 1957; Schloesser, Korst, Clatanoff, and Schilling, 1957). (Surface counting technique is further discussed in the section on iron metabolism.)

Platelet Survival.—Some information on the survival time of platelets is available. Platelets have been labelled with ^{35}S, and their survival and distribution followed in the rat (Cronkite, Bond, Robertson, and Paglia, 1957), in humans (Leeksma and Cohen, 1956), and in rats (Hjort and Paputchis, 1960), by labelling cells with di-isopropylfluorophosphonate tagged with ^{32}P (DF ^{32}P). The half-life is two to four days; those investigations using platelets labelled *in vitro* are open to the criticism that the platelets may well be altered by the preparation.

Gastro - intestinal Bleeding. — Quantitative measurement of blood loss via the gastro-intestinal tract can be made by measuring the ^{51}Cr appearing in stools after an injected dose of ^{51}Cr-labelled red cells. ^{51}Cr is not normally excreted to any

appreciable extent in the gastro-intestinal tract so that, provided the blood volume is first measured, the fraction of the given dose appearing in the stools day by day can be expressed in terms of millilitres of blood lost (Owen, Bollman, and Grindlay, 1954). Counting is conveniently done on large faecal samples in a Geiger Muller tube ring counter (Hughes Jones, 1958) although, since this type of counter is relatively insensitive to ^{51}Cr, efficiency is somewhat reduced compared with well-type scintillation counting of emulsified aliquots of the faeces. For most clinical purposes, however, the Geiger Muller ring counter is good enough, and the unpleasantness of emulsifying faeces is avoided. Quantities in the order of 3 to 5 ml. blood loss per day can be detected.

Iron Metabolism.—Much new information on iron metabolism has become available since radio-isotopes or iron have been introduced. Previously information on this topic was provided mainly by laborious balance studies and by interpretation of plasma iron concentration levels, which, though useful, are very limited in value. ^{59}Fe is the isotope in general use; it is a γ emitter, easy to count by conventional means, including external body counting, and it has a suitable half-life (46 days). A further isotope, ^{55}Fe, is available and has been used simultaneously with ^{59}Fe, but is considerably more difficult to detect (see below).

Absorption.—The method in common use is to give the tracer dose by mouth as ferrous salt in several milligrams of "carrier" (non-radioactive salt). It is said that ferrous salt is most readily absorbed (Moore, Minnich, and Roberts, 1944) and that achlorhydria does not affect absorption (Moore and Dubach, 1951; Pirzio-Biroli, Bothwell, and Finch, 1958), although recent claims have been made that the addition of ascorbic acid increases absorption even when ferrous salt is used (Bonnet, Hagedorn, and Owen, 1960). The amount absorbed is most frequently obtained by difference after counting the quantity of ^{59}Fe recovered in faeces, which must be collected for some days (Badenoch and Callender, 1954; Smith and Mallett, 1957). As usual, collection difficulties may be encountered. For clinical purposes a ring counter (Geiger Muller tubes) is suitable, and obviates the need for homogenizing the faeces.

A further method, which gives a direct measurement of the amount absorbed and is probably more accurate, uses ^{59}Fe and ^{55}Fe simultaneously. A dose of one is given by mouth, and of the other intravenously. After an interval (10–14 days) the percentage absorption is obtained from the relative amounts of the two isotopes in the red cells compared with the dose given (Bothwell, Pirzio-Biroli, and Finch, 1958). Simple measurement of plasma concentration or iron turnover rate is too dependent on additional factors to reflect absorption using one isotope only. This technique has not yet been used extensively because of the technical difficulties of counting ^{55}Fe. This isotope emits very soft x rays only, and requires the use of the gasflow proportional counter or liquid scintillation counter. When suitably prepared, the quantities of the two isotopes in a mixture can be separated by physical means (Dern, 1958), but preparation is laborious. For use in the proportional counter the iron is electroplated on to planchettes after digestion of the plasma; when liquid scintillators are used, the iron is first quantitatively electroplated on to wire and then dissolved as the perchlorate in a form suitable for solution in the phosphor-solvent system (Dern, 1960). Although the procedure is not simple it may well repay its use in future studies in intermediary iron metabolism.

While the fractional absorption of a given dose of iron is dependent on the state of the iron stores and/or erythropoietic activity at the time (Bothwell *et al.*, 1958), it is also dependent on the size of the dose, the absolute absorption increasing with the quantity given (Smith and Mallett, 1957). Thus comparative studies should always be made with a standard dose (Bonnet *et al.*, 1960). Iron absorption is decreased in steatorrhoea (Badenoch and Callender, 1954) and sometimes decreased in post-gastrectomy states (Chodos, Ross, Apt, Pollycove, and Halkett, 1957). Decreased absorption in this case may be due to unavailability of dietary iron. However, in many post-gastrectomy iron-deficiency anaemias (and probably in most other cases which appear to be unresponsive to oral iron therapy), the iron absorption is increased, the anaemia being due to occult bleeding (Smith and Mallet, 1957). Recently, interest has been shown in the problem of availability for absorption of dietary iron, and methods of labelling the iron in foodstuffs during their growth, e.g., in vegetables and eggs, are being developed (Halkett, Chodos, and Ross, 1959).

Plasma Turnover.—The curve of disappearance (or "clearance") of an injected tracer of ^{59}Fe is exponential (though not always simple). The normal range of half-clearance is 70–140 minutes (Huff, Hennessy, Austin, Garcia, Roberts, and Lawrence, 1950) and will depend on the size of the plasma iron pool, the exchangeable stores, and utilization rate of the bone marrow and other tissues (Huff *et al.*, 1950). Thus, in both poly-

cythaemia vera and iron-deficiency anaemia the disappearance rate will be high, in the first place because the marrow utilization is increased, and in the second place because although marrow utilization may be low, the plasma pool is low and the turnover rate, therefore, still high. The pathways of disappearance are not all clearly defined ; it is probable that the iron stores cannot be looked upon as a single compartment dynamically, and although such iron depots as the myoglobin do not appear to be part of the pool (West, Hahn, Clark, and Chappelle, 1952) in the normal state, it is clear that iron deficiency affects not only the haemoglobin pool, which is probably the largest single fraction of the total iron (Huff and Judd, 1956), but other iron-dependent functions as well. In chronic severe iron deficiency the skin and nails at least suffer, and it appears that depletion of iron in these is delayed until the total circulatory iron is considerably diminished. In abnormal states, then, some compartments of the pool may be avid for iron when normally equilibration with them is very slow. Such considerations make assessment of bone marrow reactivity, in terms of iron utilization, difficult and inaccurate (see below).

The disappearance rate is increased in haemolytic anaemia and in pernicious anaemia as well as iron deficiency and polycythaemia ; it is decreased in bone marrow aplasia and most acute anaemias (Fig. 1). Considerations already discussed make it clear that plasma iron turnover would give a better idea of utilization than half-clearance ; this can be calculated from the half-clearance and the quantity of the total plasma pool, which can be calculated from the plasma volume and plasma iron concentration (Huff *et al.*, 1950). This is usually expressed as plasma iron turnover per day, but since the disappearance rate is very variable over a short period (Bothwell and Mallett, 1955) the results may be considerably in error.

Erythropoiesis.—The curve of appearance of iron in red blood cells after an intravenous injection of ^{59}Fe is an inverse exponential approaching equilibrium (80–90% of the injected iron) 10 to 16 days after injection. This will be achieved more or less rapidly according to how far the actual turnover of the marrow deviates from the normal, but the value attained will be affected also if the rate of return of ^{59}Fe to the pool due to destruction of red blood cells is significant. Maximum utilization will always be achieved rapidly in iron deficiency in spite of obviously lowered haemopoietic activity, since the rate and degree of utilization will be

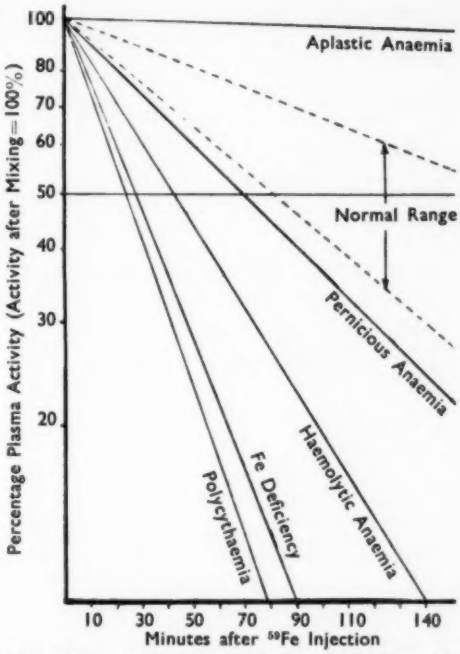


FIG. 1.—Disappearance curves after intravenous injections of ^{59}Fe. Normal range for 50% disappearance (shown on graph) is 70 to 140 minutes.

affected by the size of the plasma and storage pool. Haemolytic anaemias often show a lowered apparent utilization (in spite of obviously increased bone marrow reactivity), presumably due to the random nature of the haemolytic process. Typical utilization curves are shown in Fig. 2. The curve marked " polycythaemia " would also serve as an illustration of Fe deficiency, and that marked " renal failure " would serve for any marrow hypoplasia and for increased haemolysis. Relating the fraction of the dose appearing in the red cell mass at equilibrium to the plasma turnover rate gives a somewhat better index (Huff *et al.*, 1950), but this introduces the errors in iron turnover estimation previously discussed. Further indices, all empirical, have been suggested, such as relating the rate of appearance of labelled red cells to maximum utilization of ^{59}Fe (Joske, McAlister, and Prankerd, 1956), but none gives an entirely satisfactory measure of erythropoietic activity. One of the principal difficulties lies in the inability to determine the fraction of iron which is utilized for production of red blood cells ; a large quantity of the body iron is not concerned with haemoglobin, and the behaviour of this portion, especially

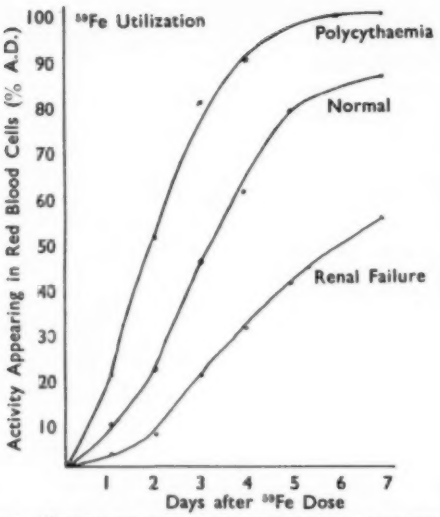


FIG. 2.—^{59}Fe utilization curves. Quantity of radioactivity appearing in red cells after dose of ^{59}Fe. Normal utilization (70–90% of administered dose) achieved in seven to 10 days. Full utilization would not appear to be achieved if considerable haemolysis were taking place. "Polycythaemia" represents typical response in high erythropoietic rates, "renal failure" typical of low rates. Fe deficiency, in spite of low erythropoietic rate, would show rapid and complete utilization because of smallness of the iron pool.

in disease, is virtually unknown. Other factors may become of major importance in disease states; one is that reticulocytes take up iron directly from the plasma (Belcher and Courtenay, 1959).

Combined ^{51}Cr and ^{59}Fe Investigations.—Simultaneous measurement of marrow reactivity and red cell survival is comparatively simple with conventional well type scintillation crystals (Weinstein and Beutler, 1955), and several workers have investigated common anaemias in this way. Uraemic anaemia is shown to be due to lowered marrow reactivity, together with a progressive shortening of cell survival time (Joske *et al.*, 1956); in leukaemia the anaemia may be due to decreased marrow reactivity and/or a shortened cell survival (Wetherley-Mein *et al.*, 1958); in pernicious anaemia the effective erythropoiesis is reduced in spite of a high plasma iron turnover, and the red cell survival is reduced (Finch, Coleman, Motulsky, Donohue, and Reiff, 1956). For clinical purposes a technique which will give semi-quantitative data on marrow reactivity and cell survival in seven to 10 days has been worked out (Wetherley-Mein *et al.*, 1958; Bailey and Prankerd, 1958).

Surface Counting Technique.—^{59}Fe emits γ rays of sufficient energy for external detection. A directional scintillation counter placed over an organ will measure changes in the concentration of ^{59}Fe within it, but the measurement will not be quantitative, due to the size and shape of the organ relative to the counter, the proximity of other organs in which the concentration may be changing, the general scatter of γ rays by the tissue, and the concentration of the isotope in the blood perfusing the organ (Huff, Elmlinger, Garcia, Oda, Cockrell, and Lawrence, 1951). The sacrum is chosen as a suitable site for marrow concentration measurement, and the heart represents blood concentration; the other sites commonly measured are liver and spleen. Considerable qualitative information is available from following the counts over these organs with time. The counts over each organ are crudely corrected for blood activity represented by counts over the heart (Wetherley-Mein *et al.*, 1956) and a curve representing activity for each organ is plotted against time. In normals maximum activity is present in the marrow at about 24 hours; thereafter the marrow activity falls and the blood level, previously having dropped, rises once more as the ^{59}Fe moves into the circulation tagged to red blood cells. The pattern of events in abnormal states, such as marrow aplasia, leukaemia, and myelofibrosis, have been clearly shown (Wetherley-Mein *et al.*, 1958), and their clinical significance discussed.

Typical curves are shown in Fig. 3. The high figures for concentration in the liver and spleen are not generally associated with extramedullary haemopoiesis, although this of course does occur in these conditions. It is presumed that large non-erythrocytogenic sources of Fe uptake are involved which are not normally present.

Comment.—The development of the techniques discussed above has led to an improved concept of the dynamics of erythropoietic turnover. The static view of the relation of marrow activity, total red blood cell (and haemoglobin) pool, and survival of red blood cells, had given rise to the chaotic "classifications" of anaemia which still survive in many textbooks. Most abnormal states can now be interpreted in terms of the interaction of these variables, and a more rational approach to disurbed mechanisms in blood diseases not yet worked out is to be expected.

B_{12} Absorption.—^{58}Co-labelled B_{12} is now almost universally used, the vitamin being produced biosynthetically using *Streptomyces griseus* (Radiochemical Centre, Amersham). ^{58}Co

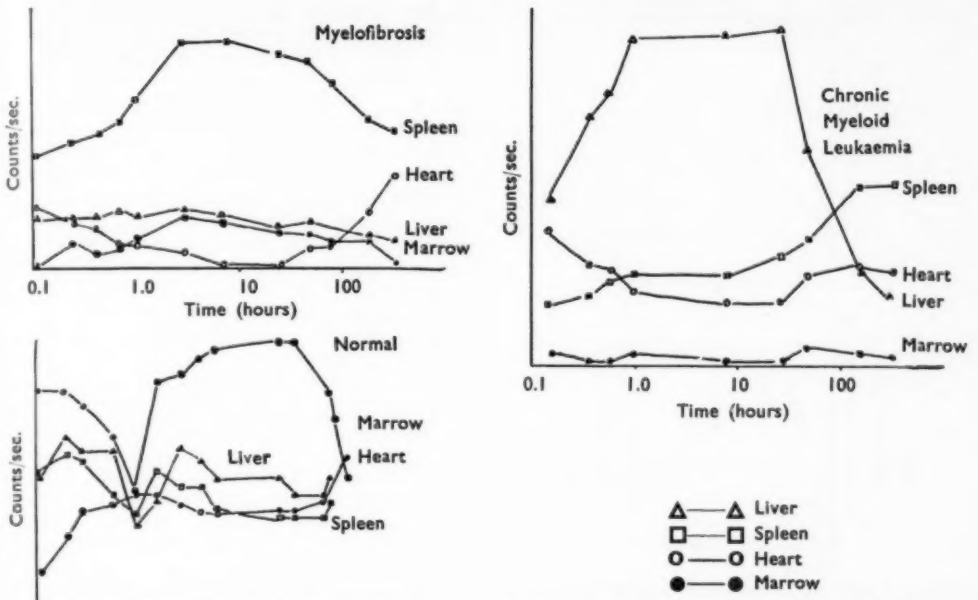


FIG. 3.—^{59}Fe surface counting graphs showing distribution in organs of injected ^{59}Fe with time. The "organ content" curves are crudely corrected for contained blood activity by subtracting the "heart" count, assuming the "heart" to represent blood. Marrow activity from count over sacrum, giving a crude measure of bone marrow reactivity. Similar procedures are used to estimate sequestration of red cells in spleen in, e.g., hypersplenism, using ^{51}Cr as red cell label.

has the advantage of a shorter half-life (71 days) over ^{60}Co (5.3 years) which was used in earlier work. The vitamin tends to be destroyed by auto-irradiation, but freeze-drying prevents this. Supplies from the Radiochemical Centre are therefore in this form. When the stock quantity is dissolved for dispensing as tracer doses, however, degradation must be expected, so that small quantities only should be obtained and used as quickly as possible.

The fraction of B_{12} which is absorbed varies with the dose given, and a standard quantity (0.5–1.0 μg.) should be administered. Reasonably accurate measurement can be made using less than 1.0 μc. The tracer is given after the patient has fasted overnight.

Three main methods for measuring absorption are used: (1) measurement of faecal excretion of ^{58}Co, and therefore, by difference, the fraction absorbed (Heinle, Welch, Scharf, Meacham, and Prusoff, 1952); (2) excretion in the urine after "saturation" by a parenteral dose of unlabelled B_{12} (Schilling, 1953, 1955; Ellenbogen, Williams, Rabiner, and Lichtman, 1955); and (3) by measurement of hepatic uptake (Glass, Boyd, Gellin, and Stephanson, 1954; Glass, Boyd, and Gellin, 1955a; Glass, Pack, and Mersheimer, 1955b; Booth and Mollin, 1956).

In the first method faeces are collected and counted until consecutive stools contain less than 1% of the administered dose. This is conveniently done in the Geiger Muller ring counter previously mentioned (Veall and Vetter, 1952). The difficulties are the usual ones where collection of faeces over several days is required. The urine excretion method requires that a "flushing" or "saturation" dose of B_{12} be given parenterally at the same time as the oral tracer dose. Urine is then collected over the next 24 hours and an aliquot measured in a well-type scintillation counter. The flushing dose ensures that a measurable proportion of the absorbed labelled vitamin is excreted, but haematologically it may "queer the pitch" for further investigation in cases of doubt. Urine collection troubles may of course be experienced. Concentration of the ^{58}Co in the urine can be achieved quite simply (Buchholz, 1958) by adsorption on charcoal, thus improving sensitivity, and allowing a reduction in dose. There is some evidence that parenteral flushing itself depresses B_{12} absorption (Baker and Mollin, 1955a; Callender and Evans, 1955), and renal excretory failure may give anomalous results. Measuring hepatic uptake by surface counting with a directional scintillation counter is simple, but quantitative measurements are not possible due to

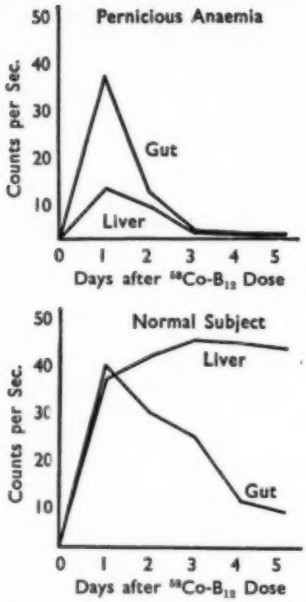


FIG. 4.—Comparison of surface activity over liver and gut in normal patients and patients with pernicious anaemia. Liver activity is the average of counts at two sites over the liver, and gut activity average of four fixed sites over the abdomen. Sodium iodide directional scintillator used: no collimator.

variations in anatomy, except perhaps by direct comparison with a subsequent intravenous dose. In pernicious anaemia, however, the activity over the liver, after time has been allowed for the gut activity to fall by excretion, is practically undetectable whereas in normal absorption a significant count is present for many months (Glass *et al.*, 1955b). Clinically the method may be useful as an out-patient screening test, or in cases of faecal or urinary incontinence, which occasionally occurs in subacute combined degeneration of the cord. Typical curves for a normal subject and a patient with pernicious anaemia are shown in Fig. 4.

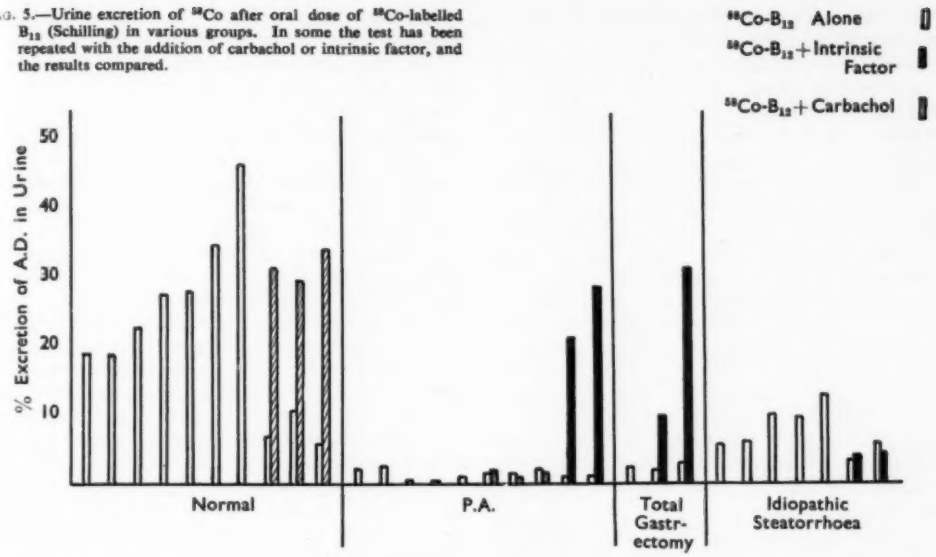
Plasma activity levels are usually not accurate enough for diagnosis using B_{12} at the specific activity usually obtainable, and in the doses normally used, but some studies have been done (Booth and Mollin, 1956) and more information on turnover and utilization could be obtained using material of a high specific activity.

Experiments have shown that B_{12} absorption is greatly reduced or absent in pernicious anaemia, that absorption is restored to normal provided intrinsic factor is given with B_{12}, and that the fraction absorbed is, over a short range, proportional to the amount of intrinsic factor given. Thus assay of intrinsic factor is possible (Baker and Mollin, 1955b; Callender, Turnbull, and Wakisaka, 1954) in patients without endogenous intrinsic factor. Hepatic turnover of B_{12} is very slow (Glass *et al.*, 1955b) as would be expected from the delayed appearance of megaloblastic anaemia following total gastrectomy and of haematological relapse when B_{12} therapy is stopped. The renal excretion of B_{12} appears to be more rapid than its uptake by the liver, which, even when depleted, appears to be slow.

In total gastrectomy absorption is similar to that in pernicious anaemia, and is likewise restored by intrinsic factor (Halsted, Gasster, and Drenick, 1954; Heinle *et al.*, 1952). In some normal fasting patients it appears that intrinsic factor may not be secreted in sufficient quantities to ensure absorption of a tracer dose; these are distinguished from abnormals by stimulation of gastric secretion by carbachol (Baker and Mollin, 1955a) whereupon absorption is restored. Absorption failure in steatorrhoea due to various causes may not be complete, as in pernicious anaemia, but neither is it restored by the addition of intrinsic factor. Fig. 5 gives typical data for normals and patients with pernicious anaemia, after total gastrectomy, and with idiopathic steatorrhoea. Some of the examples shown include the response to intrinsic factor or carbachol.

Autoradiography.—Sections or smears of tissue are suitably fixed and stained and then overlaid with a prepared film of emulsion backed with gelatin (Pelc, 1956) or coated with a fluid emulsion (Bélanger and Leblond, 1946). β particles produce blackening of grain, and the resolution depends on the characteristics of the film, the length of exposure, development, and the β-particle range. Quite accurate quantitative results can be obtained and the technique has considerable histochemical potentialities (Lajtha and Oliver, 1959). For these results, however, considerable attention to technique is necessary. Generally speaking, the dose of an isotope required in order to obtain reasonable labelling is quite high, and few studies *in vivo*, except on the thyroid, have been done. Some work on blood *in vitro* (Ellis, Lajtha, and Oliver, 1955; Boyd, Casarett, Altman, Noonan, and Salomon, 1948) is accumulating, and a very large literature on animals, in particular with the leucocyte series (Perry, Craddock, Paul, and Lawrence, 1959; Everett, Rheinhardt, and Yoffey, 1960; Perry, Craddock, Ventzke, Crepaldi, and Lawrence, 1959; Yoffey, Hanks, and Kelly, 1958).

FIG. 5.—Urine excretion of ^{58}Co after oral dose of ^{58}Co-labelled B_{12} (Schilling) in various groups. In some the test has been repeated with the addition of carbachol or intrinsic factor, and the results compared.



Many of the recent applications involve tritiated thymidine which is incorporated into nuclear protein. Tritium, due to its very short β particle range, gives very high resolution, and since a high tracer dose is allowable, it may be assumed that some results *in vivo* will soon be forthcoming.

Chemical Pathology

Body Fluids and Electrolytes.—Considerable information on the body composition of electrolytes and fluids is now available in normal and diseased states. Balance studies had previously been made on turnover rates, and measurement of fluid compartments using antipyrine (total body water), inulin (E.C.F.), and dyes (plasma volume) had been well established. The former are tedious, almost impossible except in a metabolic ward, and cumulative errors of measurement of input and output are very easily made; no information on the size of the pool is available. The latter are classical indicator dilution techniques, and depend on specific properties of limitation to certain fluid compartments. In order to distinguish them chemically they must of necessity be foreign to the compartment measured, and may not necessarily be distributed normally. Nevertheless, the techniques are well established, and in the case of antipyrine space, estimation of the antipyrine (chemically rather difficult) is facilitated by the use of ^{131}I-labelled 4-iodo-antipyrine (Talso, Lahr, Spafford, Ferenzi, and Jackson, 1955).

Since suitable isotopes have been available, it has been possible to measure the total pools of the common electrolytes, within certain important limitations.

It is clear that the body represents a more or less complicated system of "compartments" with which equilibrium may be attained to differing degrees. Various assumptions must be made about the dilution curve of the isotope with time. It is assumed that distribution is uniform throughout the pool at the time when the measured sample is taken; the assumption is made on the basis that the dilution curve has levelled out. This takes about 12 hours in the case of sodium (Miller, Munro, and Wilson, 1957), but the levelling out is usually only relative, the steep fall of the curve being succeeded by a shallower, slower one. The point at which the sample is taken will represent an average, for that time, of the whole body, but not necessarily equilibrium for each compartment. Thus, the figure obtained is approximate and represents a fraction only of the total sodium being measured. It is therefore necessary to adopt the same procedure from case to case in order to compare results, although in certain circumstances where there is a gross change in the size (and possibly other factors, such as permeability) of the compartments compared one with another, the assumptions made for normals may not be valid in disease. For instance, in oedema, ascites, and in cardiac failure, the time for flattening of the

dilution curve is about twice as long (O'Meara, Birkenfeld, Gotch, and Edelman, 1957).

It is customary to express the calculated figure for sodium or potassium content as "total exchangeable" sodium (T.E.S.) or potassium (T.E.K.) due to the above considerations. From carcass analysis T.E.S. represents about 70% of the total sodium in the body, much of the rest being relatively inaccessible in the bones (Forbes and Lewis, 1956) although over a long period equilibrium is reached with most of it. Total exchangeable potassium represents about 85% of total potassium as compared with estimation by measurement of the naturally-occurring ^{40}K in the body using whole-body scintillation counters (Rundo and Sagild, 1955). Equilibrium of potassium is much slower than of sodium, partly because, unlike sodium, most of the potassium is in cells, and partly because the low plasma concentration of potassium renders equilibrium almost entirely dependent on rate of blood flow (Black, Davies, and Emery, 1955).

Sodium space, T.E.S., and T.E.K. are measured with ^{24}Na (half-life 15.0 hours) and ^{42}K (half-life 12.4 hours). Other Na isotopes have unsuitably long or short half-lives; there are no other suitable K isotopes. The techniques for separate counting of ^{24}Na (Miller *et al.*, 1957) and ^{42}K (Corsa, Olney, Steenburg, Ball, and Moore, 1950) are well established.

Exchangeable Chloride, E.C.F., and Total Body Water.—No suitable radioisotope of chlorine exists (^{38}Cl, half-life 37 minutes, ^{36}Cl, half life 3×10^5 years) so that ^{82}Br (half-life 36 hours) is used. Certain tissues show different concentrations of chloride and bromide, but the error is probably not great (Staffurth and Birchall, 1960; Reid, Forbes, Bondurant, and Etheridge, 1956). Excretion rates of chloride and bromide by the kidney differ considerably, so that plasma samples are counted rather than urine. Total bromide is rarely particularly informative; ^{82}Br space is more commonly used as a measure of E.C.F. Comparison of E.C.F. estimates using large, chemically inert molecules (inulin, mannitol, sucrose) and ^{82}Br ("bromide" space) shows considerable difference, the ^{82}Br space giving a figure of about 17% of body weight, and that of the large molecule methods 15% (Elkinton and Danowski, 1955). Various reasons are possible: the large molecules may not properly equilibrate with the E.C.F.; bromide space includes some intracellular bromide, the amount being governed by the Donnan effect. (It is usual to apply a correction factor for this, and for ^{82}Br in the red cells (Ljungren, 1957; Manery, 1954).) In addition, ^{82}Br space probably measures (and overmeasures) fluid contents of the gut.

Pragmatically, however, although the concept of E.C.F. is explicit, the relation of the derived estimate to whatever is the absolute value is not clear, nor is it particularly important. It is better to accept the values given by the particular method used, remembering that the methods are not strictly comparable one with another. ^{82}Br space is a function of E.C.F. and varies in the expected way with changes in E.C.F. Its main advantage over other methods lies in the speed of (apparent) equilibration and the simplicity of measurement.

Total body water measurements using tritiated water are simple, equilibrium is reached in a few hours, and measurement is made on cumulative urine excretion, thus obviating the need for the repeated venepunctures of the antipyrine method. The values obtained are somewhat higher than for antipyrine (Prentice, Siri, Berlin, Hyde, Parsons, Joiner, and Lawrence, 1952), presumably due to the fact that ^3H measures exchangeable hydrogen in, e.g., hydroxyl groups. Certainly after initial rapid equilibration, there is a slower, more prolonged disappearance rate, and the concentration of tritium rises slightly in the liver.

The counting of ^3H, previously difficult, is simpler since the advent of liquid scintillation counting (see above). ^3H in urine presents no difficulties provided the urine is first decolorized by adsorption with charcoal. Plasma ^3H may be counted after previous separation of plasma water from its solutes by distillation or by precipitation.

Total body water in adults is about 55 to 60% of body weight, according to the amount of fat. Body water apparently bears a constant relation to "lean body mass" and this has given rise to a considerable literature in which various absolute quantities, e.g., red cell mass (Muldowney, 1957), are related to lean body mass, which is in turn derived by an approximation from the total body water. Unless specific information on lean or fat mass is required, it seems logical to refer other quantities to total body water and leave it at that. Certainly total body water is a much better index than body weight, height, or surface area, all widely used indices. Other quantities, such as cardiac output, oxygen consumption, are more reasonably quoted in terms of total body water, and, since ^3H measurement is simple and rapid, in future this might be done.

Intracellular fluid space cannot be derived directly, but is obtained by difference between total body water and bromide space. The total exchangeable sodium and potassium likewise have greater significance when related to body water

and bromide space. The average values for T.E.S. and T.E.K. in normals are: for sodium, males 43 mEq./kg. body weight, and females 40 mEq./kg. (Moore, 1946; Moore, McMurrey, Parker, and Magnus, 1956), and for potassium, males 45 mEq./kg. body weight, and females 35 mEq./kg. (Aikawa, Harrell, and Eisenberg, 1952; Blainey, Cooke, Quinton, and Scott, 1954; Corsa *et al.*, 1950). The ranges quoted are large; this is presumably due to the variation in proportion of fat to lean both in normals of the same sex and between male and female. Reference of these values to total body water would almost certainly give a narrower range for normal values.

Since for most investigations it is desirable to measure all the parameters mentioned above, a number of techniques have been developed for simultaneous measurement of T.E.S., T.E.K., and bromide space. Total body water presents no problem since the half-life of ^{3}H is 12.3 years and it is necessary merely to wait for the decay of the other isotopes, all of them with short half-lives, before counting the tritium in samples or to separate the water by some method. Separation of the others, since their half-lives are similar, must be done by chemical means (James, Brooks, Edelman, Olney, and Moore, 1954; Wittig and Raff, 1951; Munro, Renschler, and Wilson, 1958; Arons, Vanderlinde, and Solomon, 1954), by physical means (Tait and Williams, 1952; Robinson, Arons, and Solomon, 1955; Flear, Cawley, Quinton, and Cooke, 1958; Munro *et al.*, 1958), or some convenient combination of both. A comparison of both techniques is given by Munro *et al.* (1958).

Metabolic Turnover.—Information on turnover rates of sodium and chlorine is obtainable because suitable isotopes (^{22}Na, half-life 2.6 years; ^{36}Cl, half-life 4.4×10^{5} years) are available. The metabolic half-life of these elements is about 10 days. Since ^{36}Cl is a β emitter, measurement of turnover must be made by urine and faecal collection (Ray, Burch, and Threefoot, 1952), but by using a suitable whole body counter (Veall *et al.*, 1955a) ^{22}Na turnover can be measured by direct estimation of the amount of the isotope remaining in the body. Thus measuring excretion is unnecessary; given suitable isotopes it may be possible to adapt this method to other metabolic processes.

The advent of more sensitive whole body counters (Anderson, Schuch, Perrings, and Langham, 1955; Langham, 1958) has made possible measurement of body content of the naturally occurring isotope ^{40}K, the quantity of which is very closely related to lean body mass. Changes in lean body mass, and indirectly overall protein metabolism in the human body in disease states, should therefore be measurable in the future. Fat/lean proportions of pigs, produced commercially for different purposes in the bacon and pork products industries, are already measured in this way.

Plasma Proteins.—These are labelled in two ways: the proteins are autogenously labelled by giving ^{14}C-labelled precursors (Campbell, Cuthbertson, Matthews, and McFarlane, 1956; Abdou and Tarver, 1951; Volwiler, Goldsworthy, MacMartin, Wood, MacKay, and Fremont-Smith, 1955), or by labelling *in vitro* protein preparations for injection (McFarlane, 1956).

The plasma proteins are collectively labelled by the former method, so that differentiation is difficult; turnover and half-life studies are affected by re-utilization of amino-acids in protein anabolism (Armstrong, Kukral, Hershman, McLeod, and Wolter, 1954; Masouredis and Beeckmans, 1955; Volwiler *et al.*, 1955). In general, doses of ^{14}C or ^{35}S are comparatively large and not very suitable for human studies.

Labelling *in vitro* is open to criticism on the grounds that the protein may be altered in preparation and may therefore not behave in the same way as the protein it is meant to tag (Berson, Yalow, Shreiber, and Post, 1953; Volwiler *et al.*, 1955); it has been shown that molecules heavily laden with ^{131}I do not have normal physical characteristics. Improved methods of preparation (McFarlane, 1956) have reduced the number of iodine atoms per molecule and the evidence is that ^{131}I-labelled proteins behave in a similar way to autogenously produced ^{14}C-labelled ones. This can be assumed to be true of ^{131}I-labelled human serum albumin when carefully prepared since good quality serum albumin is readily available, but probably not of the more complex globulins and fibrinogen.

Calculation of distribution, pool size, and turnover using ^{131}I-H.S.A. depends on assumptions that the compartments through which the albumin is distributed are defined, and that the system is in equilibrium over the period of measurement (Campbell *et al.*, 1956). In practice, the thyroid is blocked to iodine uptake by previous administration of Lugol's iodine, and, after intravenous administration of the dose of ^{131}I-H.S.A., blood samples are taken over several days and plasma activity measured. Urine is collected continuously and the excreted ^{131}I measured. (In cases of urinary protein loss, e.g., the nephrotic syndrome, urinary protein is calculated separately from the free iodine in the urine.)

Values for the plasma pool, plasma metabolic turnover rate, total body pool, and total body turnover rate can be obtained. Mere information on half-life, without regard to the size of the pool, is misleading (Bauman, Rothschild, Yalow, and Berson, 1955), and some earlier work should be treated with reserve. Nevertheless, the normal range of plasma ^{131}I-H.S.A. half-life appears to be somewhere between five and nine days, and that of the total albumin pool about 16 to 21 days. In a few cases where there appears to be a defect of albumin production, the clumsily named "idiopathic ananabolic hypoproteinaemia," the metabolic half-life is as long as 35 days (Gordon, Bartter, and Waldmann, 1959), while in some cases of accelerated protein loss (nephrotic syndrome, exudative enteropathy, "hypercatabolic" hypoproteinaemia) it is as short as two to four days (Gordon, 1959; Gordon *et al.*, 1959; Blahd, Fields, and Goldman, 1955; Gitlin, Janeway, and Farr, 1956). Chronic liver disease and possibly starvation result in a reduced albumin pool and a slow rate of production of protein has been demonstrated (Bauer, Blahd, Fields, and Getchell, 1954). Other failures of protein synthesis have been shown in congenital afibrinogenaemia and agammaglobulinaemia (Gitlin and Borges, 1953). Changes in distribution of the pool have been examined in ascites and oedema (Berson and Yalow, 1954) and the multicompartment systems involved treated mathematically.

Excessive loss of protein can occur in the gut, but except in diarrhoea, due, for example, to ulcerative colitis (Steinfeld, Davidson, and Gordon, 1957), such loss is not easily measured because of complete or partial reabsorption of the ^{131}I after protein destruction within the gut. Recent investigation using ^{131}I-labelled polyvinylpyrrolidone (P.V.P.) (Gordon, 1958), which has a molecular structure and size analogous to H.S.A. but is not destroyed in the gut, has shown that some hypoproteinaemic conditions exist due to an abnormal loss of protein in the stomach or small intestine (Gordon *et al.*, 1959; Gordon, 1959). Some cases described are characterized by abnormally low total plasma proteins, high E.S.R., and high plasma cholesterol, and some have a condition similar to chronic hypertrophic gastritis. More investigation of gut conditions of this sort is required; ^{131}I-P.V.P. might also help in settling the question of whether active excretion of protein into the lower gut can occur in, e.g., steatorrhoea.

Fat Absorption.—Triolein and oleic acid can be iodinated without great difficulty in the laboratory and a suitable preparation for absorption tests obtained. That obtainable from the Radiochemical Centre, Amersham, is iodinated to completion and unlabelled triolein added to give the required specific activity. It is thus expensive, but for ordinary work is not necessary. As a diagnostic test a tracer dose of iodinated triolein is given by mouth and the fraction absorbed obtained by difference after counting the activity excreted in the faeces (Lubran and Pearson, 1958), or blood samples obtained and the plasma activity measured (Baylin, Sanders, Isley, Shingleton, Hymans, Johnston, and Ruffin, 1955; Beres, Wenger, and Kirsner, 1957; McKenna, Bourne, and Matzko, 1957). The former method requires faecal collection with its attendant difficulties, and contamination with urine cannot be allowed due to excretion of iodine after endogenous splitting of fat.

The results, however, agree fairly well with those of the more laborious chemically determined faecal fat excretion and measurement is simpler. Table I shows the figures for ^{131}I excretion after

TABLE I

COMPARISON OF DAILY FAECAL FAT EXCRETION (CONVENTIONAL METHOD OF ESTIMATION) WITH CUMULATIVE EXCRETION OF ^{131}I AFTER ADMINISTRATION OF ^{131}I-TRIOLEIN

	Faecal Fat in g./Day (Normal up to 7 g.)	A.D. ^{131}I in Faeces (%)
Normal	3·0	0·4
.. .. ..	5·0	4·6
Crohn's disease ..	72·0	76·0
Jejunal resection ..	13·4	12·0
Ulcerative colitis* ..	10·9	1·3

*Note anomalous result for "ulcerative colitis."

giving ^{131}I triolein, compared with the simultaneous chemical measurement of fat excretion in a small group of patients. It will be seen that one result is anomalous and is not easily explained. More data on the correlation of these two tests in different fat-absorption states should be available soon (Vaughan Jones, 1960). There appears to be little difference whether the dose is taken alone, with a meal, or with a quantity of fat; the easiest way is to enclose it in a gelatin capsule, since some correction is otherwise necessary for the portion of the dose left after drinking an emulsion. It is also less unpleasant to take. In normals not more than about 5% of the administered dose is recovered in the faeces.

Blood levels of ^{131}I are often equivocal. The normal levels of activity are not very high and there is evidence that ^{131}I is rapidly split off (Beres *et al.*, 1957), presumably due to the instability of the iodinated bond, since ^{14}C-labelled triolein disappears less quickly. There is evidence, too (van

Handel and Zilversmit, 1958), that ^{131}I-triolein may be split during absorption, based on comparison of ^{131}I- and ^{14}C-triolein absorption, and thus interpretation of plasma concentration curves is very difficult. The value of plasma levels is that a peak figure is reached in a few hours, and the test may therefore be used as a screen to exclude normals.

In pancreatic disease and steatorrhoea due to other causes, e.g., idiopathic, post-gastrectomy, absorption of ^{131}I-triolein is reduced. In pancreatic failure (when not due to fibrocystic disease) the absorption of ^{131}I-oleic acid is normal, however, and this is in contrast to other causes of steatorrhoea, in which the ^{131}I-oleic acid absorption is impaired along with the ^{131}I-triolein (Turner, Parker, Coffey, and Duffy, 1956; Shingleton, Isley, Floyd, Sanders, Baylin, Postlethwait, and Ruffin, 1957). In fibrocystic disease of the pancreas, the ^{131}I-oleic acid absorption may be impaired (Reemtsma, Malm, and Barker, 1957) which accords with the more recently held view that the condition is one involving the gut and probably other systems as well as the pancreas. Table II illustrates the differences in absorption in three typical cases.

TABLE II

COMPARISON OF DAILY FAECAL FAT EXCRETION, AND ^{131}I FROM ^{131}I-TRIOLEIN AND ^{131}I-OLEIC ACIDS

	Faecal Fat Excretion (g./Day)	Faecal ^{131}I Excretion after ^{131}I-Triolein (% A.D.)	Faecal ^{131}I Excretion after ^{131}I-Oleic Acid (% A.D.)
Normal	4·2	2·0	1·3
Pancreatectomy ..	35·2	95·8	5·7
Idiopathic steatorrhoea	21·8	37·3	28·1

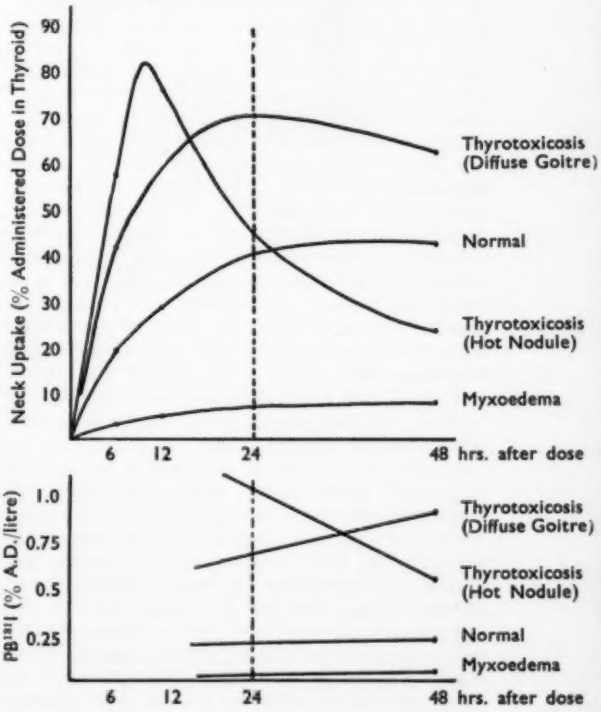
Recently ^{82}Br-triolein has been used and, once more, labelling in the laboratory is quite simple (Lubran and Corsini, 1959). Results seem to be comparable to those using the ^{131}I label, and since its half-life is short (36 hours) and its γ remission more energetic, it may have advantages from the point of view of repeatability. Since ^{131}I and ^{82}Br are readily separable physically, simultaneous tests of absorption, e.g., oleic acid and triolein, should be possible with much saving in time.

The effect on absorption of, e.g., pancreatin, bile salts, is not very clear at present, due mainly to the laborious fat balances previously necessary, and ^{82}Br-labelled fats and fatty acids could well be used in such investigations.

Thyroid Metabolism

Investigations of thyroid function are numerous and a voluminous literature has accumulated. Radioactive iodine is taken up by the thyroid gland, incorporated into thyroid hormone, and secreted by the gland. The iodine on injection or ingestion is distributed rapidly throughout a large fluid space, and its disappearance from this space is a function of renal excretion (G.F.R.) and the avidity of the thyroid for iodine. An inverse relationship therefore exists between the amount taken up by the thyroid and the amount excreted in the urine; in general, but not always, a high uptake and low urine excretion over a given period mean that the gland is overactive and therefore that the patient is thyrotoxic. Similarly the appearance in the blood of a high concentration of radioactive protein-bound iodine over a given period is indicative of a high iodine turnover rate in the gland, in general indicating hyperthyroidism. Measurement of urine excretion of radioactive iodine, of the uptake of iodine by the thyroid, or of the concentration of protein-bound radioactive iodine might

FIG. 6.—General curves for ^{131}I neck uptake and PB^{131}I with time, in various conditions. Line at 24 hours indicates point at which most routine readings of these are taken. Note that at 24 hours the neck uptake in a nodular goitre may have fallen into the normal range. PB^{131}I will usually be abnormally high, however.



each be expected to provide an indication of the functional state ; each does, but individually they present considerable difficulties and results may be equivocal. Fig. 6 shows the general pattern of uptake with time of the thyroid gland in various conditions together with the corresponding values for $PB^{131}I$. It should be noted that the patterns for the " hot " nodule and the toxic diffuse goitre are different (for reasons discussed later) and that in particular the 24-hour thyroid uptake level in the former may be indistinguishable from the normal.

Measurement of urine excretion is simple, but the best information is provided by splitting up a collection period over 24 to 48 hours, and this presents difficulties ; it can never be certain that all urine is collected. In addition, renal or cardiac failure may impair renal excretion, and although some correction may be made for this (Fraser, Hobson, Arnott, and Emery, 1953) results are sometimes difficult to interpret. By itself, correlation with the clinical state of the patient is not, in our hands, very good (Keating, Power, Berkson, and Haines, 1947 ; Skanse, 1949).

Measurement of thyroid uptake depends on surface counting, and the techniques employed are almost as numerous as the laboratories employing them. A " directional " scintillation counter placed at a standard distance to " view " the thyroid, and comparison of the count thus obtained with a standard, is widely used, but differences in anatomy of the gland and the scattering effect of the tissues introduce errors. More satisfactory geometry, minimizing variations in anatomy, is achieved using a combination of counters (Geiger Muller tubes) which " see " a cylindrical section of neck (Freedberg, Chamovitz, Ureles, and VanDilla, 1950). Comparison is then made with a standard. The use of a standard is avoided by comparing counts over the neck with those from non-thyroid tissue, e.g., of the thigh, expressing these as a ratio (Pochin, 1950 ; Foote and MacLagan, 1951). This method is particularly useful for repeated counts over a short period after administration of radio-iodine.

Uptake figures are usually expressed as the percentage uptake at a given time (Keating, Haines, Power, and Williams, 1950) after administration of a dose (24 hours is a convenient time after an oral dose) or as a curve of uptake rate over a few hours after an oral or (better) intravenous dose. Due to the wide variation in methods used, results from a given laboratory can only be fairly compared within the range of values obtained in that laboratory. Uptake curves show wide variation with respect to time and concentration and give little information in absolute terms. This is due to several factors. First, the mass of the gland is virtually unmeasurable : although attempts have been made to do this by multiplane surface counting, the effort is hardly worth while. Second, the gland frequently does not function equally throughout its anatomical mass (Sinclair, Abbatt, Farran, Harriss, and Lamerton, 1956) even in the apparently " diffuse " goitres of Graves's disease ; and third, since the amount of iodine in the gland at any time is a function of uptake, release (as hormone) and storage (as thyroglobulin), the " uptake " figure is not simple and may be distorted very considerably. For example, a small functional quantity of gland may be working to capacity to maintain either a normal or abnormal output of thyroid hormone, with little or no storage, while a large functional quantity may be producing exactly the same results with a much lower turnover rate per unit of gland, and with considerable capacity for storage. The reasons for these differences are obscure ; although the former tendency is exemplified by the " hot nodule," even the apparently homogeneously active gland often shows areas of varying behaviour.

The value of thyroid uptake in the diagnosis of thyroid disease is undoubtedly high. Fig. 7 shows the distribution of thyroid uptake in a number of consecutive routine diagnostic tests, a clinical assessment having been made of each patient previously by the same observer. There is a considerable region of doubt, however, and a number of clinically doubtful cases remain

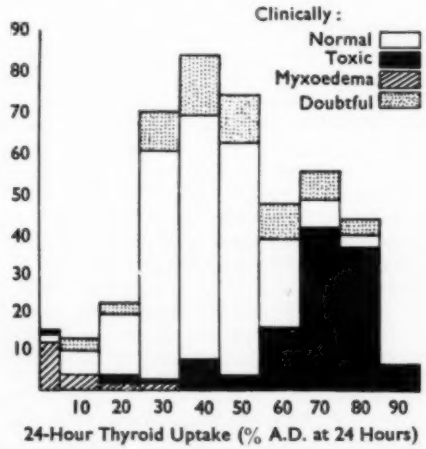


FIG. 7.—Distribution of patients in their clinical gradings, according to their ^{131}I neck uptakes. Note considerable overlap of grades.

unelucidated. It is convenient usually to give ^{131}I by mouth and measure the uptake at 24 hours, but the more recent introduction of ^{132}I with a half-life of 2.3 hours has made possible assessment of thyroid function with a much smaller radiation dose. Its short half-life precludes urine and P.B.I. measurement and allows only plotting the rate of uptake over a few hours, so it is probably better to inject the ^{132}I intravenously. The ^{132}I is eluted, when necessary, directly from a ^{132}Te column (supplied by the Radiochemical Centre, Amersham). Neck-thigh ratio measurements (Halnan, 1958) are very suitable, providing useful screening tests which will protect the majority from the greater radiation of a ^{131}I tracer dose. Very small doses allow repetition of the test when necessary, and thyroid assessment in infants and children may be considered feasible.

Information on the hormone output of the thyroid depends on measurement of the quantity of ^{131}I appearing in the protein-bound fraction of iodine in the plasma. In practice this is measured by counting the total quantity of radioactivity in the protein precipitated from a sample of plasma taken, say, 24 hours after a tracer dose. The ^{131}I is in fact bound to the α_1 globulin fraction and to a lesser extent to the albumin (Horst and Rösler, 1953). The concentration of ^{131}I will depend on the rate of release of hormone by the gland, the rate of utilization, the amount of available protein for binding, and the total pool of thyroid hormone.

In pregnancy the available binding protein fraction rises, and so does the bound thyroid hormone, presumably passively. This is the probable cause of the goitre and high ^{131}I uptake in pregnancy. The rate of utilization of hormone does not increase, however. Conversely, in the nephrotic syndrome the binding protein falls, but the fraction of free hormone is normal. Thus it appears that the protein-bound iodine is not consistently proportionate to the total hormonal pool, and therefore the $PB^{131}I$ is not always simply related to the total hormone turnover.

The $PB^{131}I$ is frequently used as a measure of thyroid function, either by itself or as a "conversion" ratio (Clark, Moe, and Adams, 1949) with the unbound plasma ^{131}I representing free iodine. This depends on the fact that as the $PB^{131}I$ rises the free ^{131}I falls; therefore the ratio rises in thyrotoxicosis and falls in myxoedema. Cardiac or renal failure, or iodine-deficient goitre, will, however, give anomalous results.

Fig. 8 shows the distribution of $PB^{131}I$ results in a number of consecutive routine diagnostic tests, a clinical assessment having been made of each patient previously by the same observer. As with the figure showing thyroid uptake results (Fig. 7), the overlap is rather large. Many of the clinically "doubtful" patients remain doubtful.

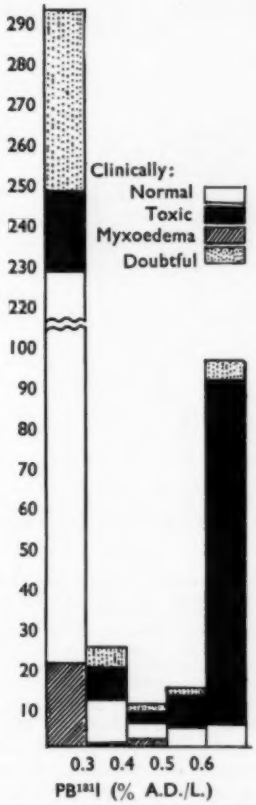


FIG. 8.—Distribution of patients in their clinical gradings, according to their $PB^{131}I$ values. Note considerable overlap of grades.

Probably the best results are obtained by measuring both the thyroid uptake and the $PB^{131}I$ in each patient. In this laboratory differentiation (in patients previously assessed clinically by the same observer) was obtained in 90% of nearly 450 consecutive routine uptakes (Fig. 9). It will be seen that the great majority of those diagnosed as thyrotoxic give values for thyroid uptake of more than 60% of the administered dose and for $PB^{131}I$ of above 0.3% per litre. (It must be stressed that these values are probably unique to this laboratory, and that the calibration will be different for different laboratories.) It will also be seen that

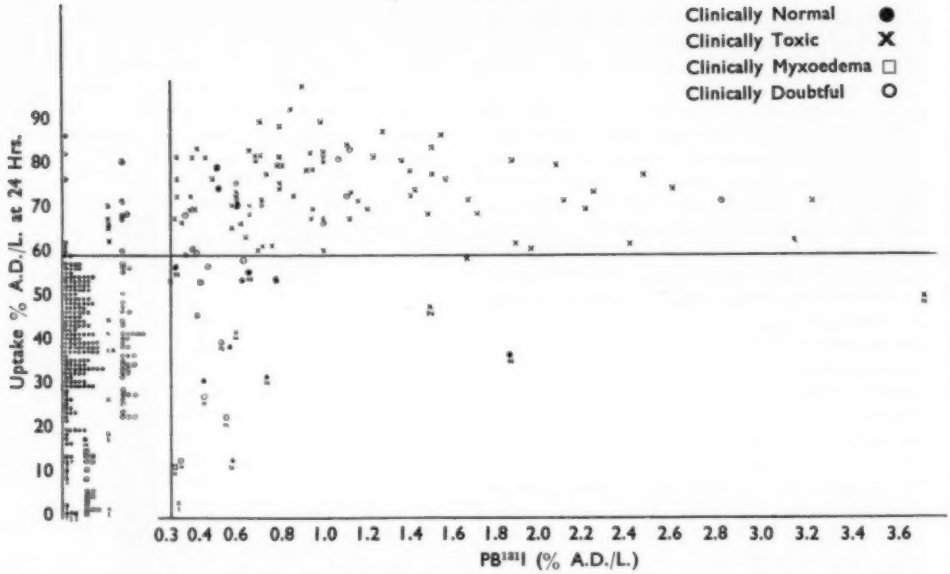


FIG. 9.—Uptakes of patients (clinically graded) plotted against their $PB^{131}I$ readings. A very large majority (90%) can be discriminated, normals lying below the 60% uptake level and to the left of 0.3% $PB^{131}I$ level, thyrotoxics lying above and to the right of these levels. Myxoedema patients lie to the left of 0.3% $PB^{131}I$ level and below 20% uptake level. Points to the left of 0.3% $PB^{131}I$ line are plotted on the uptake axis only. Points scattered widely from these limits are often explicable (see text); those labelled "N" are nodular goitres, "H" are Hashimoto's disease; "I" and "T" have recently had exogenous iodine and thyroid respectively. Some of the points lying above the 60% uptake level, but to the left of 0.3% $PB^{131}I$ level, are brought into the normal range by thyroid suppression tests.

some of those giving apparently anomalous results are due to explicable causes. For instance, in some of the cases of Hashimoto's disease, the uptake is very low while the $PB^{131}I$ is abnormally high; the 24-hour uptake figure may be low in comparison with the $PB^{131}I$ figure in nodular thyrotoxicosis (although the peak may be much higher shortly after the dose); some results are invalidated by previous unsuspected administration of iodine or thyroid (two of these latter patients were thyroid "addicts").

A few of the results show very high thyroid uptakes but with $PB^{131}I$ figures which are in the normal range. This behaviour is sometimes observed in cases which later develop a clearly thyrotoxic picture, but sometimes the simple non-toxic goitre gives these results. On the assumption that in simple goitre the high uptake is due to a relative iodine deficiency, a standard test (Burrell and Fraser, 1957) has been applied which discriminates between the two conditions. A small quantity of potassium iodide is given daily for a week, followed after an interval by another uptake test. In simple goitre the uptake is reduced compared with that of the first test, the iodine deficiency being made up by the iodide; in thyrotoxicosis the high uptake continues. A further discriminatory test is also available; the high uptake of the simple goitre is suppressed by a short course of tri-iodothyronine (a few days), while that of the hyperthyroid gland is not (Werner and Spooner, 1955). This test is also easily performed and has the advantages of a shorter time scale. Some of the anomalous results shown in Fig. 11 were clarified in this way.

Recent work (Hamolsky, Golodetz, and Freedberg, 1959; Robbins, 1959) has demonstrated that when ^{131}I-tri-iodothyronine is incubated and shaken with blood, it is divided between the cells and plasma in a ratio which increases in thyrotoxicosis and decreases in hypothyroidism. In a large group of experiments a clear distinction is shown, although not very adequately related to the clinical state of the patients used. The explanation is presumably that the remaining unbound plasma protein takes up ^{131}I-tri-iodothyronine preferentially, and that the excess is bound on to red blood cells. When the available protein is reduced (because a large part is already bound to the patients' hormone) the red blood cell binding s

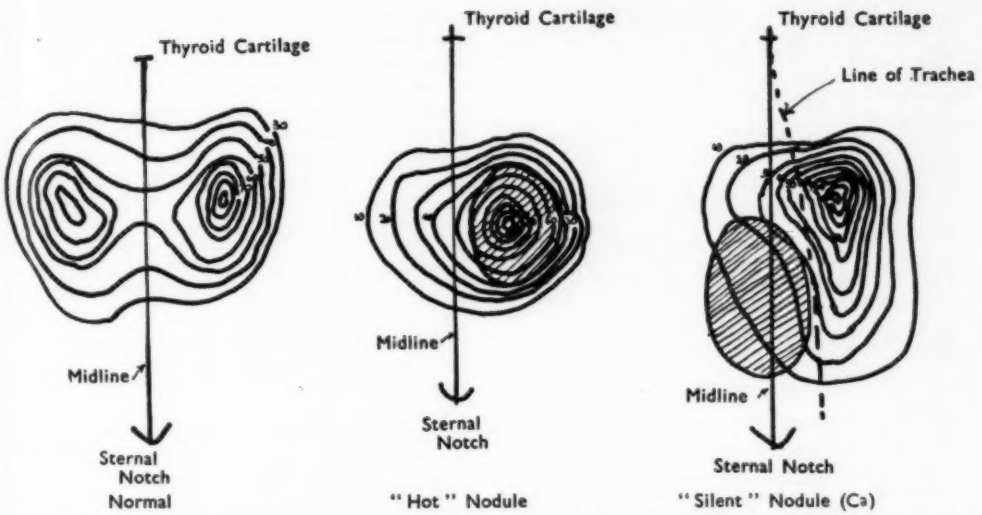


FIG. 10.—Typical examples of surface scans over (1) " hot nodule," (2) " silent nodule " (thyroid carcinoma), and (3) a normal gland. The isocount lines show 10% decrements from the point of highest count.

large and vice versa. Further evaluation is necessary; the system appears to be sensitive to CO_2 (and therefore *p*H ?), but this method may well offer a useful screening test which is simple and avoids the giving of radioactivity to the patients. Its usefulness in infants and children is obvious.

Hashimoto's disease and other forms of thyroiditis in general show lowish or low uptakes, although a high uptake is occasionally seen in Hashimoto's disease. Characteristically ^{131}I is expelled from a Hashimoto goitre if a small quantity (400 mg.) of potassium perchlorate is given by mouth shortly after the ^{131}I dose. This contrasts with the behaviour of a simple goitre in which the ^{131}I uptake continues unimpaired (Morgans and Trotter, 1957). The reason is obscure, but presumably some abnormality of organic binding of the iodine must be involved. Typical behaviour in Hashimoto's compared with that in simple goitre is shown in Fig. 10. The PB^{131}I tends to be high compared with uptake in Hashimoto's disease; this is possibly related to a high turnover rate in a small remaining quantity of functioning gland. The sensitivity of the ^{131}I tests to pick out these cases is not very good; where there is doubt thyroglobulin precipitin tests should be done.

Congenital and familial abnormalities of thyroid function have been shown but usually are very rare. Various metabolic blocks have been demonstrated (McGirr and Hutchison, 1953; Stanbury and Querido, 1956) including iodine deficiency goitre due, presumably, to the absence of an enzyme which normally de-iodinates the monoiodotyrosine freed when thyroglobulin is broken down (Stanbury, Kassenaar, Meijer, and Terpstra, 1955).

Thyroid carcinoma tissue is characterized by a low or absent uptake, but the rest of the gland in which the carcinoma arises may behave normally or may even be overactive. Surface scanning may reveal a " silent " nodule if it is big enough compared with the adjacent normal gland, and it is wise to consider any silent area or nodule as a possible neoplasm. However, small neoplastic areas are not likely to be revealed by scanning, since resolution of the distribution of activity is not good enough. Further difficulties arise due to the fact that, although the gland may appear homogeneous anatomically, it very often is not so functionally.

Treatment of thyroid carcinoma with ^{131}I depends on the ability of carcinoma tissue to concentrate radioactive iodine; it is almost always negligible when normally functioning thyroid tissue is present. Ability to concentrate is (not surprisingly) related to the degree of differentiation histologically, and generally speaking the results are disappointing. " Silent " nodules may also be due to degenerative cysts and some appear to consist of normal quiescent gland tissue.

" Active " nodules may cover a range of functional behaviour from the normal to extremely

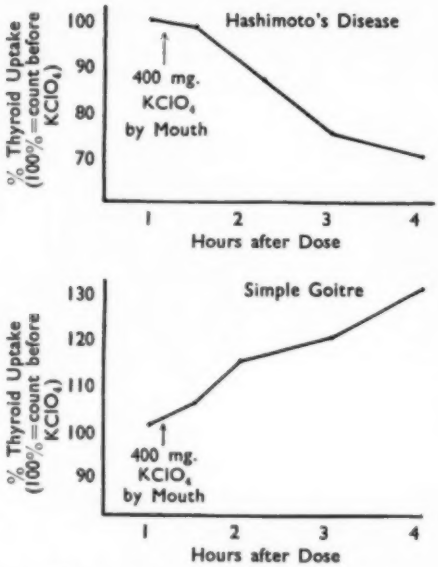

% Thyroid Uptake (100% = count before $KClO_4$)
Hashimoto's Disease
400 mg. $KClO_4$ by Mouth
Hours after Dose
Simple Goitre
400 mg. $KClO_4$ by Mouth
Hours after Dose

FIG. 11.—^{131}I neck counts from a patient with a simple goitre and with Hashimoto's disease after an oral dose of $KClO_4$. The ^{131}I is given by mouth one hour earlier, and the readings after the $KClO_4$ compared with that immediately before, which is taken as 100%.

high concentration. Such "hot" nodules may secrete sufficient hormone to produce thyrotoxicosis; on the other hand, a similar appearance on the scan can be given by a localized mass of thyroid which by rapid turnover and low thyroglobulin storage is maintaining normal hormonal output, the rest of the gland contributing little. Such nodular behaviour, by no means uncommon, often presents a difficult diagnostic problem, as the quantitative difference between the nodule producing the disease and that maintaining normal function is not easily measured.

The difference in uptake and $PB^{131}I$ curves between the diffuse goitre and the nodule is shown in Fig. 6. Typical scans of a normal gland, a hot nodule, and a large thyroid carcinoma are shown in Fig. 11.

General Considerations

Use of Radioisotopes in Diagnosis.—In view of the concern felt by a public which is rapidly becoming aware of the danger of ionizing radiations, it is probably better not to carry out radioisotope studies unless there is a clear case for them. In the general run of investigation or diagnosis it is quite often reasonable to dispense with an isotope "test" when an equally informative conventional test is available. For instance, when documentary evidence is needed "for the record" in a clear case of thyrotoxicosis, it seems proper to estimate the B.M.R. rather than ^{131}I uptake, since the more sensitive differentiation possible with the latter is not needed. Similarly, most of the electrolyte imbalance states arising in disease can be inferred from clinical observation and the plasma electrolyte concentration figures, so that measurement of total exchangeable sodium or potassium is not necessary. This attitude is justified further by the undoubted truth that the general level of ionizing radiation received by the public during its "patient" life is increasing, and a tracer study should be thought of in the same terms as a radiological procedure when considering the question, Is this justified? Finally, there is at the moment little legislation regarding the use of, and disposal of, radioactive isotopes, and an attitude of caution in their use is less likely to make any subsequent statutory control unduly restrictive. For all these reasons the aim should be to increase the sensitivity of the counting procedure so as to decrease the tracer dose, but retaining the same degree of accuracy.

Record of Tracer and Treatment Doses.—It appears essential to have a clear and permanent record in the patient's notes of all tracer and treatment doses given which should be entered at the time the dose is given, preferably in some prominent place which is a fixture. This is necessary for several reasons. First, it provides a complete record of the accumulation of doses that the patient may receive during his hospital life; this could be considerable over many years. Second, such a record, easily referred to, will prevent the possibility (not so very unlikely!) of a second tracer dose being given for a second investigation while the first is still in progress; and third, a clear record is available if the patient comes to the post-mortem room, so that the pathologist and his staff can take the necessary precautions, and not be exposed to radiation of which they might otherwise be unaware. This is not usually a risk with tracer doses, but is of very considerable importance should the patient die soon after a treatment dose. Tracer dose levels can be of importance when they are concentrated in a particular organ, such as ^{131}I in the thyroid, when that tissue is manipulated during histological preparation.

In this hospital a gummed ticket, suitably coloured red, is stuck into the inside cover of the patient's folder, when he has his first tracer dose, and the particulars of the dose and all subsequent

doses are entered on the ticket as they are given. The further arrangement is made that should any patient die who has a red ticket in his folder, the cadaver is automatically given a prominent distinguishing mark before leaving the ward. This warns post-mortem room staff of possible radiation risks which can be confirmed or denied by reference to the date and dose record displayed on the red ticket.

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Numerous texts have appeared in recent years, most of them of uneven quality. Probably the best, because the most up to date, comprehensive, and critical, is *Radioisotope Techniques in Clinical Research and Diagnosis*, by N. Veall and H. Vetter (1958), Butterworth, London. Theoretical and practical aspects of general techniques are described in detail.

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SHORT-TERM ASSESSMENT OF RED BLOOD CELL SURVIVAL USING ^{51}Cr

BY

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^{51}Cr techniques for the study of red blood cell survival over long periods in the steady state are now well established (Ebaugh, Emerson, and Ross, 1953; Mollison and Veall, 1955; Hughes Jones and Mollison, 1956). This paper presents the results of an investigation of the validity of a ^{51}Cr study not exceeding 10 days which is well suited to a combination with a ^{59}Fe technique for the rapid simultaneous assessment of red cell production and destruction where haematological conditions may show fairly rapid changes.

Material and Methods

Ten normal subjects and 12 patients with normal ^{51}Cr red cell survival were investigated. Twenty millilitres of the subject's blood, withdrawn into heparin, was centrifuged at 1,800 r.p.m. for 15 minutes. The plasma was removed and 100 μc. ^{51}Cr as $Na_2{}^{51}CrO_4$ added to the packed cells. After standing at room temperature for 30 minutes the cells were washed once, suspended in isotonic saline, and then reinjected into the subject. A blood sample was taken 15 minutes after reinjection for determination of blood volume, and afterwards about 12.5 ml. 0.1% sodium citrate in isotonic saline containing 10 μc. of $^{59}FeCl_3$ was given intravenously to allow the subsequent determination of plasma ^{59}Fe clearance and turnover, the utilization of ^{59}Fe for red blood cell production, and the ^{59}Fe surface counting pattern (Wetherley-Mein, Hutt, Langmead, and Hill, 1956). ^{51}Cr activity was determined in blood samples taken in a standard sequence (disposition A, Table I) during 10 days following reinjection of the tagged cells.

The component ^{51}Cr and ^{59}Fe activities in blood samples containing both isotopes were determined in a well-type scintillation counter, the scaler being equipped with a variable voltage discriminator, thus allowing the inclusion or exclusion of ^{51}Cr activity in the total counting rate.

Determination of Rate of ^{51}Cr Loss.—Starting 48 hours after the injection of ^{51}Cr cells the values representing ^{51}Cr activity over seven to 10 days are plotted logarithmically against time on a linear scale and the best-fitting straight line through the points is drawn. From the slope of this line the rate ^{51}Cr loss (percentage loss per day) may be calculated. It is convenient to calibrate a protractor in terms of daily percentage ^{51}Cr loss against a standard time scale.

Results

From these pooled residual error variances, we estimated the overall error of a single careful determination of relative ^{51}Cr activity to correspond to a coefficient of variation of $\pm 2.85\%$; of this we could identify a quantity estimated as $\pm 1.91\%$ as referable to the actual counting, which we termed "scaler error." The residual difference ($\pm 2.15\%$) thus represents all other handling errors (Ingram, Langmead, and Jones, to be published).

We examined two series of results in the normal subjects and patients with no evidence of shortened red cell survival and found no evidence of any real differences in the ^{51}Cr activity of serial blood samples other than those assignable to the steady removal of the isotope from the blood. This investigation also confirmed the empirical justification for the expression of normal ^{51}Cr loss in exponential terms over short periods of time (see Discussion). In 10 normal subjects we obtained a mean rate of loss of ^{51}Cr from the circulation of 7.99% per day, with the real variation between subjects giving a coefficient of variation of $\pm 30\%$. Our mean rate agrees well with the figure of about 2.75% per day derived from the data for days 2 to 10 recorded by Mollison and Veall (1955) in 11 normal subjects. We also found that observed short-term fluctuations in the background activity, i.e., within a

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day, did not detectably contribute to our experimental errors.

From these analyses we were able to compute the critical values for the least rate of ^{51}Cr loss which could confidently be regarded as abnormal when derived from various dispositions of blood samples in a 10-day period. These values are given in the right-hand column of Table I and

TABLE I
DISPOSITIONS OF SAMPLES

	Disposition of Blood Samples: Days after Injection of ^{51}Cr (Day 0)									Limiting Value for ^{51}Cr Slopes (% Loss of Activity per Day)
	2	3	4	5	6	7	8	9	10	
(A)	××	×				×			××	5·1
(B)	××	×			×	××				5·4
(C)	××	×		×	××					5·6
(D)	×	×	×	×	×	×				5·7

× indicates that a blood sample should be taken.
×× indicates that two separate samples should be taken, one immediately after the other, from different veins.

show the least slope which for each disposition of samples defines the lower limit of abnormal ^{51}Cr loss with the same confidence as 5.1% per day in our standard 10-day sequence (disposition A). On this basis, a similar grouping of six samples over six days (disposition C) must yield a rate of ^{51}Cr loss greater than 5.6% per day to be regarded as abnormal.

As with the measurement of any constant rate of change, ^{51}Cr loss is progressively more accurately determined as the time over which it is observed is lengthened. Within a given period the accuracy is also affected by the disposition in time of the readings obtained since its precision is governed by the moments of these readings about the midpoint. Thus, for a given number of blood samples over 10 days, accuracy is improved by bunching the samples at each end of this period.

The gain in accuracy obtained by bunching the samples at the ends of the study is seen by comparing dispositions B and D.

The left-hand column in Table I gives three dispositions of samples found useful in clinical situations where blood samples cannot be taken in the standard 10-day sequence (A).

Discussion

In normal subjects correction of the ^{51}Cr survival curve for a uniform rate of chromium elution from red cells fails to give the linear slope that is taken to represent true normal cell survival (Hughes Jones and Mollison, 1956). This causes difficulty in interpreting survival curves that do not differ greatly from normal. One might use the early part of the curve to obtain an estimate of mean cell life (Dornhorst, 1951; Hughes Jones and Mollison, 1956). However, since the "early loss" of ^{51}Cr makes interpretation of the first part of the curve unreliable, the mean cell life is in practice often the least valid deduction that can be made from a 10-day study.

Alternatively, it would be possible to express the results in terms of half-life of ^{51}Cr in the circulation uncorrected for elution. To this approach may be offered two objections. First, in slow regressions, determination of the half-life point may require considerable extrapolation. Secondly, this use of the concept of half-life is meaningful only if the regression is strictly exponential to the point of near extinction. As Dornhorst (1951) showed, this is true only of red blood cell populations subject to random destruction so intense that age-loss can virtually be ignored.

To meet the objections given above, and yet keep the study short, we have adopted the following method of interpretation. Total ^{51}Cr loss is treated as a single exponential function over the period 48 hours to 10 days, and the rate of loss (percentage loss ^{51}Cr per day) is determined. For normal subjects it has been shown that if the ^{51}Cr survival is plotted in this way the departure from linearity, if the period of "early loss" (0–48 hours) is excluded, is slight over the first 50 days (Mollison and Veall, 1955). Certainly over the short period we have chosen the points are well fitted by a straight line on semi-logarithmic paper. Normal curves fall so slowly that, even were they linear, they would not materially deviate from an exponential over this portion of the curve, whereas rapidly falling curves are likely to represent random destruction and will therefore approximate to exponentials. Thus in each case a measure of rate of loss can be obtained using a 10-day curve. This use of the data facilitates comparison of rates of ^{51}Cr loss in the same subject over two or more short periods of observation, e.g., before and after treatment.

In certain haemolytic syndromes there is evidence that early ^{51}Cr elution may be decreased and this may mask shortened red blood cell life (Mollison, 1959; Jones, N. F., unpublished). It is thus not legitimate to fix an upper limit for the normal rate of ^{51}Cr loss over a period as short as 10 days. We therefore approached this problem empirically. In 10 normal subjects, from whom samples were obtained in the standard sequence over days 2 to 10, ^{51}Cr loss did not exceed 4.25% per day. In

patients with undoubted haemolysis shown by conventional haematological methods, we have observed over the same period rates of ^{51}Cr loss varying from 5.5% to 19% per day. On the basis of this experience it appeared that, for this disposition of blood samples, rates of ^{51}Cr loss greater than 5.0% per day could be regarded as abnormal.

An analysis of our results in the group of normal subjects and an investigation of the experimental error showed that only one in 20 normal subjects would be expected to show rates of ^{51}Cr loss exceeding 5.1% per day if the samples were taken in our standard sequence. As this figure agreed closely with the 5.0% per day arrived at empirically, we have taken 5.1% per day to be the critical value above which rates of ^{51}Cr loss are abnormal. It must be recognized that a rate of ^{51}Cr loss of less than 5.1% per day over this short period does not necessarily imply normal red blood cell survival; but it may then be inferred that haemolysis is not contributing greatly to the patient's anaemia. With so short a study it is not permissible to calculate mean cell life, and when this is suspected of being only slightly shortened the period of ^{51}Cr study must be extended.

While, therefore, this 10-day technique has obvious limitations, it is extremely valuable both in clinical laboratory practice and for assessing the effects of treatment on red cell survival in rapidly changing haematological states. It may also be combined with a ^{59}Fe study of red cell production.

We wish to thank the consultant physicians and surgeons of St. Thomas's Hospital for permission to study their patients, Professor A. C. Dornhorst for his advice and criticism, and Dr. P. Armitage, of the Medical Research Council's Statistical Research Unit, London School of Hygiene and Tropical Medicine, for his advice on the statistical aspects of this investigation. The expenses of the investigation were partly borne by the Endowment Fund, St. Thomas's Hospital.

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THE ABSORPTION OF VITAMIN B_{12} IN MEGALOBLASTIC ANAEMIA ASSOCIATED WITH PREGNANCY

BY

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The absorption of $^{60}CoB_{12}$ has been studied in a group of patients suffering from megaloblastic anaemia associated with pregnancy and in a control group.

The mean urinary excretion of $^{60}CoB_{12}$ in the patients with megaloblastic anaemia is not significantly different from that of the control group. Five of the 30 patients showed a value towards the lower limit of normal, but the serum vitamin B_{12} content in this group was within the normal range.

Malabsorption of vitamin B_{12} does not appear to play a significant role in the pathogenesis of this form of megaloblastic anaemia.

The role of deficient intake, impaired absorption, and loss of anti-megaloblastic factors in the pathogenesis of megaloblastic anaemias associated with pregnancy is obscure. Recent investigations have drawn attention to absorption defects in this group. Chanarin, Anderson, and Mollin (1958) have presented evidence suggesting some impairment in the absorption of folic acid in normal or apparently normal pregnancy, and Baker (1958) finds that the common cause of megaloblastic anaemia in pregnancy in India is a vitamin B_{12} deficiency due primarily to the presence of an intestinal lesion which impairs B_{12} absorption.

In Bantu adults in South Africa megaloblastic anaemia is encountered most frequently following pregnancy (Cassel and Metz, 1958). The condition appears to differ from the megaloblastic anaemias of pregnancy in white races, in that it often presents some months after confinement. While 64% of cases occur within three months after delivery, 20% of the patients present with anaemia more than six months after confinement (Metz, 1958). The present study was undertaken to determine the role, if any, of malabsorption of vitamin B_{12} in this group.

Material and Methods

The subjects investigated were Bantu hospital patients. All had been delivered within the previous year, and were still lactating. Two groups were studied.

Controls.—These were 20 non-anaemic subjects who were convalescing from various diseases, e.g., pneumonia, not usually affecting absorption from the gastrointestinal tract. The relation to pregnancy is shown in Table I.

TABLE I

MONTH AFTER CONFINEMENT AND RESULTS OF SCHILLING TEST IN CONTROLS AND PATIENTS WITH MEGALOBLASTIC ANAEMIA

Controls			Megaloblastic Anaemias		
No.	Month after Confinement	Urinary Excretion of $^{60}CoB_{12}$ (%)	No.	Month after Confinement	Urinary Excretion of $^{60}CoB_{12}$ (%)
1	4	4·3	1	1	3·9
2	6	5·0	2	3	4·1
3	3	5·1	3	2	4·3
4	11	5·8	4	1	4·4
5	10	6·3	5	3	4·4
6	5	6·7	6	7	5·9
7	2	7·3	7	5	5·9
8	1	7·4	8	3	6·2
9	1	7·8	9	2	6·9
10	1	7·8	10	8	9·5
11	1	8·1	11	1	9·9
12	2	10·1	12	1	10·6
13	11	10·1	13	1	10·9
14	2	12·0	14	2	13·5
15	7	13·7	15	1	14·3
16	1	15·2	16	2	14·4
17	2	16·6	17	1	14·6
18	2	21·1	18	4	14·7
19	1	26·0	19	3	15·1
20	3	30·2	20	1	15·3
			21	9	19·3
			22	6	19·8
			23	4	22·2
			24	3	23·2
			25	5	24·2
			26	1	24·3
			27	2	24·4
			28	1	24·8
			29	1	30·4
			30	3	38·7
Mean		11·3	Mean		14·6
S.E.		1·5	S.E.		1·6

Patients.—These were 30 subjects suffering from megaloblastic anaemia. All showed classical or intermediate megaloblasts and giant myeloid cells in the bone marrow. The relation to pregnancy is shown in Table I.

The haematological methods employed were standard (Dacie, 1954). The absorption of radioactive vitamin B_{12} was studied by a modification of the Schilling test, as described previously (Metz, Cassel, and Lewis, 1958).

Results

Controls.—The urinary excretion after oral administration of a dose of 0.6 μg. vitamin B_{12} labelled with 0.15 μc. ^{60}Co to the control subjects ranged from 4.3 to 30.2% of the administered dose (mean value 11.3%).

Megaloblastic Anaemia.—The urinary excretion after administration of an identical dose of $^{60}CoB_{12}$ to the group of patients with megaloblastic anaemia ranged from 3.9 to 38.7% of the administered dose (mean value 14.6%). The results are detailed in Table I.

Of the 30 patients studied, five showed a urinary recovery of less than 5% of the administered dose of vitamin B_{12}, while only one of the 20 control subjects absorbed less than 5% of the dose. In our clinic normal subjects usually absorb more than 5% of the dose, while patients with Addisonian pernicious anaemia absorb less than 4% of the dose, this defective absorption being corrected by the concomitant administration of intrinsic factor. In two patients where the urinary recovery of $^{60}CoB_{12}$ was 3.9% and 4.3% respectively, the concomitant administration of a potent source of intrinsic factor together with the labelled vitamin B_{12} did not enhance the absorption of the vitamin.

With the Schilling test, failure to collect all the urine passed during the 24-hour collection period may result in spuriously low values for $^{60}CoB_{12}$ excretion. Of the five patients in the present series with a urinary excretion of less than 5% of the administered dose, the volume of urine passed was 1,300, 1,280, 1,000, 710, and 510 ml. respectively. In the first four of these patients, the serum vitamin B_{12} content was 270, 340, 450, and 150 $\mu\mu$g./ml. (In view of the small urinary output, the result of the Schilling test in the patient with a urinary volume of 510 ml. must be treated with reserve.)

Discussion

These results indicate that the absorption of vitamin B_{12} in a group of patients suffering from megaloblastic anaemia associated with pregnancy is as efficacious as that of a control group of non-anaemic subjects. The mean value for the Schilling test in the anaemic group is in fact greater than that of the control group, but the difference is not statistically significant. In the cases where the absorption of vitamin B_{12} is towards the lower limit of normal, the presence of a normal serum vitamin B_{12} content suggests that malabsorption of the vitamin does not play a significant role in the pathogenesis of this form of megaloblastic anaemia.

We wish to thank the Director, South African Institute for Medical Research, for providing the facilities for this study; Dr. E. Lester-Smith, of Glaxo Laboratories, Greenford, for generous supplies of radioactive vitamin B_{12}; Dr. D. L. Mollin, of the Department of Haematology, Postgraduate Medical School, London, for carrying out the vitamin B_{12} assays; Lederle Laboratories, New York, for supplies of an intrinsic factor preparation; and Glaxo Laboratories, Wadeville, for supplies of vitamin B_{12}.

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TISSUE REACTIONS TO ASPERGILLUS IN CASES OF HODGKIN'S DISEASE AND LEUKAEMIA

BY

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Five cases of aspergillosis complicating Hodgkin's disease and leukaemia are reported. The organs involved were: lungs (all five cases), stomach (Case 3); brain and meninges (Case 4); heart, kidneys, spleen, thyroid, and liver (Case 2). Cultures of *Aspergillus fumigatus* were obtained from the post-mortem tissues of three patients.

All the lesions in Case 2 were suppurative. The other four cases had non-suppurative lesions characterized by spreading coagulation necrosis with peripheral hyperaemia, exudation, and haemorrhage. Invasion and occlusion of blood vessels occurred frequently.

The various factors that may be responsible for the initiation and progression of the fungal infection are discussed. The available evidence suggests that *Aspergillus fumigatus* can produce toxic metabolites which are able to cause tissue necrosis and vascular damage. In patients suffering from neoplastic conditions of the lympho-reticular system, especially if steroid hormones or radiomimetic drugs are given, spreading, necrotizing lesions can develop unchecked by antibody or cellular defences.

During recent years deep (systemic) fungal infections have assumed considerable importance. The increasing use of powerful antibiotics, various chemotherapeutic agents, and steroid hormones has coincided with a rising incidence of mycoses complicating other diseases, especially neoplastic conditions of the lympho-reticular system. Before these drugs were introduced, aspergillosis usually presented as a chronic bronchopulmonary disease. In a classical monograph, Rénon (1897) described it as an occupational hazard amongst pigeon-feeders and wig-makers. Coe (1945) reported the case of a man aged 47 who had worked for 20 years in the Union Stock Yards. He noted that aspergillus is a regular inhabitant of the soil and can be isolated frequently from cereal products, unmilled grain, hay, and other stock foods. The organism was saprophytic on the grain and corn-meal used by these workers. Aspergillosis may also complicate other chronic pulmonary diseases such as carcinoma, lung abscess, tuberculosis, pneumoconiosis, and bronchiectasis (Heppleston and Gloyne, 1949; Hinson, Moon, and Plummer, 1952; Golebiowski, 1958; Finegold, Will, and Murray, 1959). When aspergillosis complicates a generalized debilitating disease such as malignant lymphoma treated with one or more of the various drugs now in use, the fungal infection is more rapidly progressive and has an increased tendency to disseminate.

The purpose of this paper is to record five additional cases of aspergillosis complicating leukaemia and Hodgkin's disease. In particular we wish to describe in some detail the tissue reactions to the fungus in this type of case. We do not propose to review the historical or mycological aspects since these have been adequately covered by other workers (Hinson *et al.*, 1952; Thom and Raper, 1945).

Material and Methods

During a period of 18 months (November, 1957, to May, 1959) aspergillosis was discovered *post mortem* in five patients at the Royal Marsden Hospital. The primary disease was Hodgkin's lymphoma in one case, acute myeloid leukaemia in three, and lymphatic leukaemia in one (Table I). Over the same period, 65 necropsies were performed on patients with leukaemia or malignant lymphoma, so that the proportion showing secondary aspergillus infection was between 7 and 8%. In no case was the diagnosis of aspergillosis made during life. *Aspergillus fumigatus* was isolated from post-mortem tissues in three patients (Cases 1, 2, and 5); swabs were taken from the lesions and inoculated on to Sabouraud's medium, subculturing being continued until a pure growth was procured (Fig. 1). In the other two cases, cultures were not obtained, but we feel that there is little doubt that the organism seen in tissue sections was an aspergillus since morphological criteria were satisfied (Torack, 1957); the hyphae were of medium width (10–15 μ), were septate,

TABLE I
SUMMARY OF PRESENT SERIES

Case No. and Laboratory Reference	Sex	Age	Predisposing Disease	Type of Tissue Response to Aspergillus	Organs Involved	Other Infections
1 PM. 108/57	F	41	Hodgkin's disease	Spreading, necrotizing	Lungs	Staphylococcal abscesses of lung; staphylococcal empyema; moniliasis pharynx and oesophagus
2 PM. 57/58	M	44	Acute myeloid leukaemia	Suppurative	Lungs, liver, kidneys, spleen, myocardium, pericardium, thyroid	—
3 PM. 83/58	M	32	Acute lymphatic leukaemia	Spreading, necrotizing	Tracheal mucosa, lungs, pharynx, stomach	—
4 PM. 5/59	F	14	Acute myeloid leukaemia	Spreading, necrotizing	Lungs, brain, meninges	—
5 PM. 32/59	M	40	" "	"	Lungs	Small staphylococcal abscesses in skin and myocardium

and showed prominent branching (Fig. 2). Typical sporing heads were found in sections prepared from the lungs of Case 1 (Fig. 3). Multiple tissue blocks were fixed in 10% buffered formalin and embedded in paraffin wax in the usual way. Sections were stained by the following methods: haematoxylin and eosin, Weigert's iron haematoxylin and van Gieson's mixture, Weigert's elastin stain, Mallory's phosphotungstic-acid-haematoxylin, the picro-Mallory and acid picro-Mallory techniques (Lendrum, 1949), silver impregnation for reticulin, the periodic-acid-Schiff technique, Gram's method, Perls' reaction for iron, and toluidine blue and alcian blue (for acid mucopolysaccharides). In addition, it was found that staining with carbol fuchsin and counterstaining with light green was a very satisfactory technique for the differential colouring of hyphae and spores.

Clinical Features

Details of treatment and white cell counts are summarized in Figs. 4 to 8. The following are brief résumés of the individual case records.

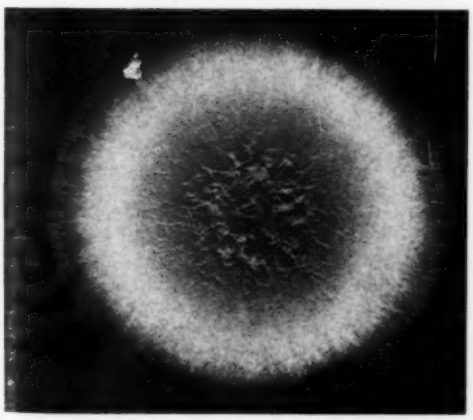

FIG. 1.—Pure culture of *Aspergillus fumigatus* isolated from Case 5. 2I•

Case 1.—A woman aged 41 years first noticed enlarged cervical lymph nodes in December, 1956. Biopsy in February, 1957, showed typical Hodgkin's disease. In March, block dissection of the cervical nodes was carried out. Radiotherapy to the mediastinum, neck, and left axilla was given in May. She remained very well until November, when she was readmitted to hospital on November 6, 1957, with a three weeks' history of fever and malaise. For a few days she was treated with aminochlorambucil without effect. Prednisolone, penicillin, and streptomycin likewise produced no improvement. Her respiration and pulse rates rose and remained high until her death on November 20, 1957.

Case 2.—A man aged 44 years was apparently in good health until Christmas, 1957, when symptoms of increasing fatigue, dyspnoea, and headaches began. A diagnosis of acute myeloid leukaemia was established, and he was treated with 6-mercaptopurine, prednisolone, and blood transfusions. His general condition remained fair until May 2, 1958, when he was readmitted with dyspnoea, cough, foul sputum, muscular tenderness, and severe general malaise. Examination showed some rales at both bases and generalized muscle tenderness. His condition deteriorated rapidly with increasing dyspnoea, pyrexia, and tachycardia until his death on May 6, 1958.

Case 3.—A man aged 32 years had complained of tiredness and dyspnoea for a few months. Investigations established the diagnosis of lymphatic leukaemia in May, 1957. At this time the spleen was palpable and there were enlarged lymph nodes in the neck and mediastinum. Treatment with 6-mercaptopurine and blood transfusions produced considerable improvement. Later in 1957 he was given methotrexate and prednisolone. Aminochlorambucil was administered in April, 1958, after his blood count was found to be rising again. During May and June further blood transfusions were needed. In July he became acutely ill with pain in the left chest, productive cough with blood-stained sputum, and purpura of the skin. His condition deteriorated rapidly, and he died on July 12, 1958.

FIG. 2

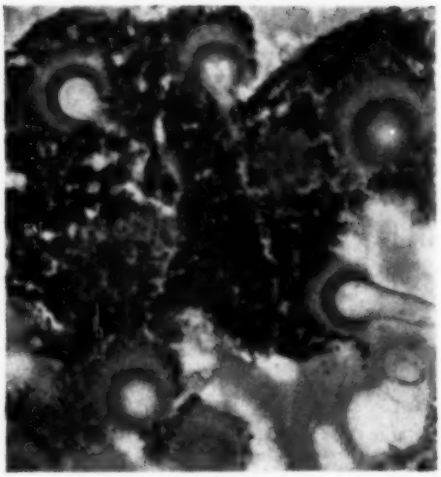

FIG. 3

FIG. 2.—Case 1. Fungal hyphae in a section of the right lung. Haematoxylin and eosin ×320.

FIG. 3.—Case 1. Sporing heads of *Aspergillus fumigatus* in a bronchial lumen. Prepared from the same tissue block as the section illustrated in Fig. 14. Spores red: hyphae, vesicles, and sterigmata green. Carbol fuchsin, light green ×320.

Case 4.—A schoolgirl aged 14 years presented with fatigue, pyrexia, a papular rash, enlarged glands in both axillae, splenomegaly, and hepatomegaly. A diagnosis of acute myeloid leukaemia was established in December, 1957. She was treated with prednisolone and 6-mercaptopurine. This produced a remission that lasted until July, 1958, when symptoms recurred. Further treatment with 6-mercaptopurine produced haematological improvement, but she still felt unwell. In December, 1958, she was readmitted to hospital in a state of general debility having suffered from haemoptysis and haematemesis. She died in January, 1959.

Case 5.—A man aged 40 years presented with profound anaemia in June, 1958. Investigations established the diagnosis of acute myeloid leukaemia. He was treated with 6-mercaptopurine, methotrexate, prednisolone, triamcinolone, and repeated blood transfusions. A persistent tachycardia was present throughout his illness. His final admission to hospital was on February 14, 1959, when he complained of increasing dyspnoea and cough with small quantities of mucoid sputum. These symptoms persisted, and three days before his death on March 10, 1959, pyrexia and a rising pulse rate developed.

Pathological Findings

Macroscopic Appearances.—We consider it unnecessary to describe detailed post-mortem findings apart from those changes which were due to the aspergillus. No definite residual Hodgkin's disease could be discovered at necropsy in Case 1. Cases 2, 3, 4, and 5 did not show any features unusual for patients dying of leukaemia, except for the fungal infections.

Four cases had non-suppurative lesions with distinctive naked-eye and microscopic features. In Case 2 the lesions were suppurative and abscesses had formed in several organs.

Non-suppurative Lesions.—The smallest pulmonary fungal lesions were firm, deep-red nodules with opaque, pale yellow centres. Such foci were scattered widely through both lungs in Cases 3 and 4. A larger lesion was found in the left lower lobe of Case 5. It consisted of a lobulated, opaque, yellowish-grey mass, irregular in outline and surrounded by a red zone of hyperaemia. Some carbon pigment was trapped in the central area, producing a black speckling. The appearances of this lesion suggested a confluence of smaller nodules; small independent foci were present in the surrounding lung (Fig. 9). In Case 1 there was a still larger area of lung involvement. The affected tissue was firm and deep red (Fig. 10). On its cut surface the lesion had indefinite margins and there were many irregular opaque yellowish patches similar to those seen in the smaller foci already described; the bronchi were moderately dilated and contained mucoid secretion and friable brownish material (shown microscopically to be fungal mycelium). These changes were found in the right lung. The left

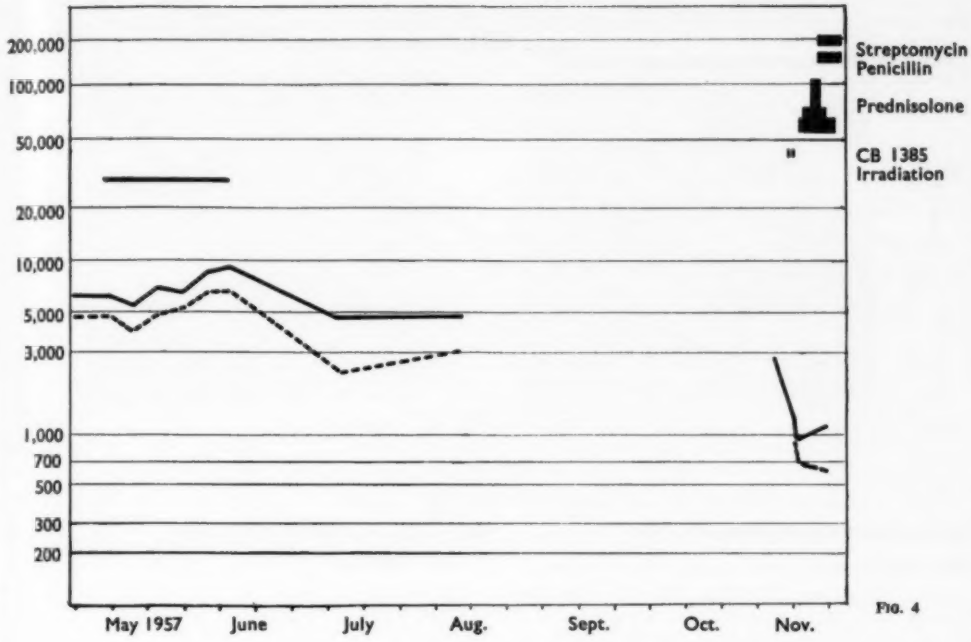

FIG. 4

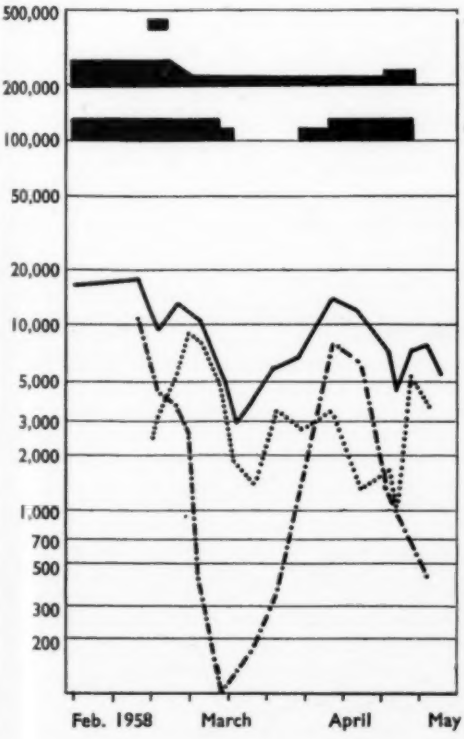

FIG. 5

FIG. 4.—Chart of white counts and treatment of Case 1. *Range of dosage:* Prednisolone 100/500/mg./day, CB 1385 (aminochlorambucil) 10 mg. i.v. Key: ——— Total white count per c.mm. – – – – Polymorphs per c.mm.

FIG. 5.—Chart of white counts and treatment of Case 2. *Range of dosage:* Prednisolone 60–30 mg./day. 6-Mercaptopurine 200–100 mg./day. Max. dose 2·5 mg./kg. Key: ——— Total white count per c.mm. ..... Polymorphs per c.mm. –·–·–·–·– "Blast" cells per c.mm.

FIG. 6.—Chart of white counts and treatment of Case 3. *Range of dosage:* NorHN$_2$, 300 mg./day. CB 1385 (aminochlorambucil), 10–5 mg./day. A.C.T.H., 50 units/day. Triamcinolone, 70–12 mg./day. Prednisolone, 60–20 mg./day. Methotrexate, 5–2.5 mg./day. 6-Mercaptopurine, 150–125 mg./day. Max. dose 2.5 mg./kg. Key: ——— Total white count per c.mm. ..... Polymorphs per c.mm. –·–·–·–·– "Blast" cells per c.mm.

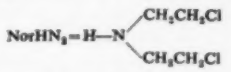

FIG. 7.—Chart of white counts and treatment of Case 4. *Range of dosage:* Methotrexate, 5 mg./day. 6-Mercaptopurine, 125–100 mg./day. Triamcinolone, 40–20 mg./day. Prednisolone, 60–5 mg./day. Key: ——— Total white count per c.mm. ..... Polymorphs per c.mm. –·–·–·– "Blast" cells per c.mm.

FIG. 8.—Chart of white counts and treatment of Case 5. *Range of dosage:* Methotrexate, 5 mg./day. Triamcinolone, 48 mg./day. Dexamethasone, 12 mg./day. Prednisolone, 18–15 mg./day. 6-Mercaptopurine, 175–125 mg./day. Max. dose 2·5 mg./kg. Key: ——— Total white cell count per c.mm. ..... Polymorphs per c.mm. –·–·–·–·– "Blast" cells per c.mm.

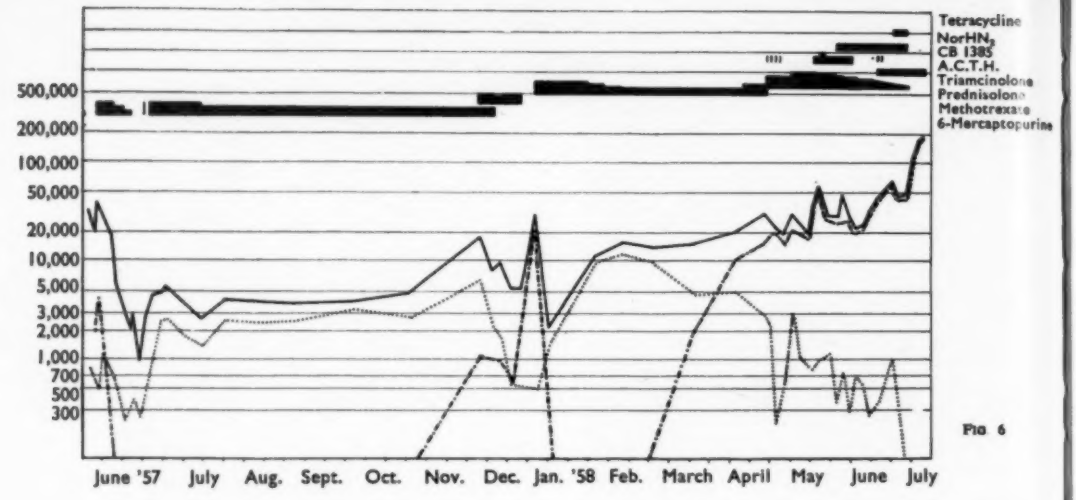

FIG. 6

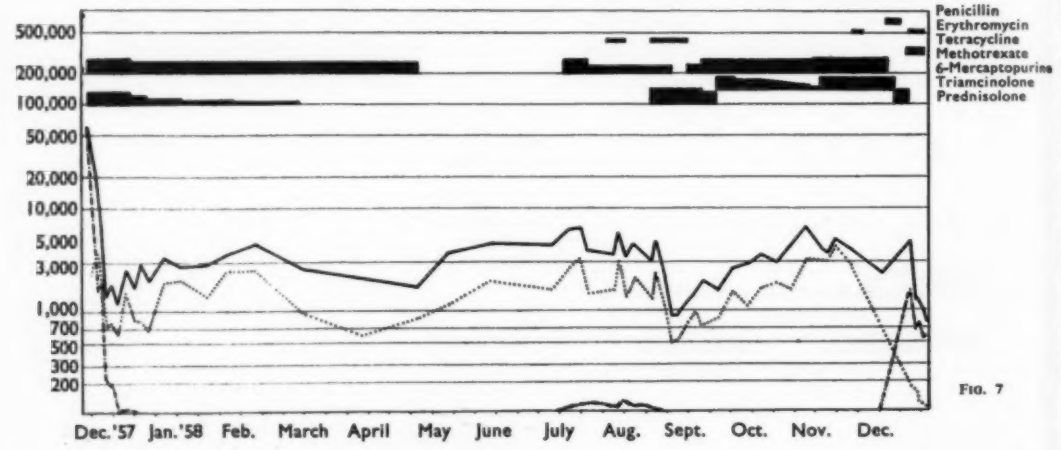

FIG. 7

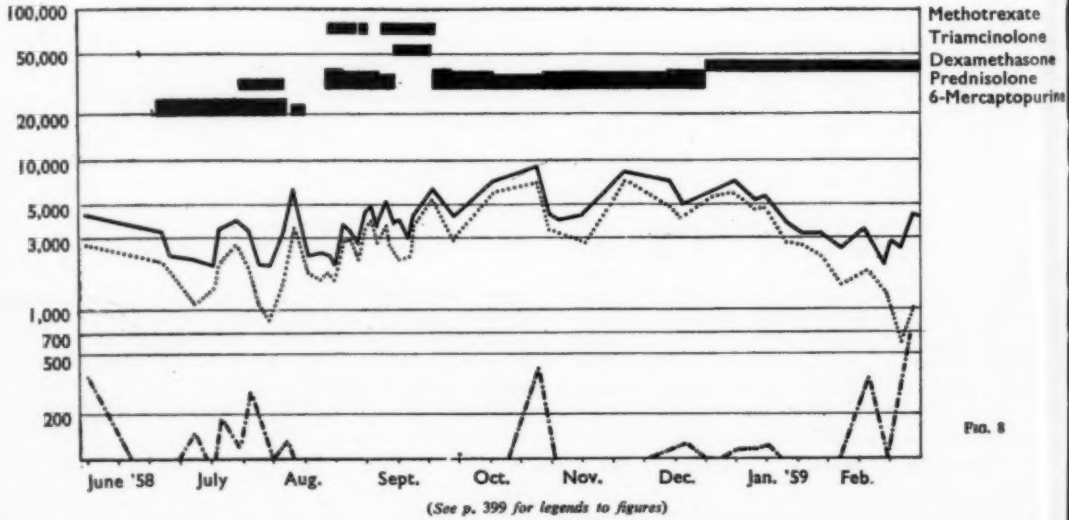

FIG. 8

(*See p. 399 for legends to figures*)

FIG. 9

FIG. 10

FIG. 9.—Case 5. Cut surface of left lower lobe showing necrotic lesion with peripheral hyperaemia. Smaller independent foci are also present.

FIG. 10.—Case 1. Cut surface of right lung showing deep red, haemorrhagic area with multiple necrotic foci.

lung contained staphylococcal abscesses and there was a small left-sided empyema. No tissue liquefaction or cavitation was seen in any of the non-suppurative lesions. In Case 3 the trachea and main bronchi showed small mucosal ulcers with yellowish bases and red borders. Similar lesions were present in the mucosa of the laryngopharynx and stomach. In Case 4 there was involvement of the brain. A few patches of haemorrhage were present beneath the pia arachnoid over the cerebral hemispheres. There were two small patches of necrosis with haemorrhage in the left parietal lobe and a tiny area of similar haemorrhagic necrosis in the upper part of the pons over its ventral aspect.

Suppurative Lesions.—Case 2 had disseminated aspergillosis with involvement of the lungs, pericardium, myocardium, spleen, kidneys, thyroid, and liver. The pulmonary lesions were small, discrete yellowish nodules distributed throughout both lungs. The pericardial sac contained about 200 ml. of blood-stained fluid, and a thick fibrinous exudate covered the serosal surfaces of the pericardium and epicardium. A few tiny abscesses, containing yellow pus, were found deep in the posterior wall of the left ventricle. Small abscesses were present in the spleen, which weighed 213 g. There were multiple cortical abscesses in the kidneys (right 283 g., left 265 g.). The upper half of the right kidney contained a deep red infarct. Both lobes of the thyroid were enlarged and deeply congested: no suppuration was observed on naked-eye examination. The liver was pale, but the fungal lesions were of microscopical dimensions only.

Histological Observations

The naked-eye changes in Cases 1, 3, 4, and 5 correspond to a characteristic microscopic pattern of necrosis without suppuration. All the lesions in Case 2 show polymorph infiltration and pus formation. The non-suppurative and suppurative types of tissue reaction will be described separately.

Non-suppurative Lesions.—Not only do these lesions fail to suppurate but they display little evidence of any inflammatory cell response. We have designated this type of reaction as "spreading-necrotizing," a term which emphasizes the chief features. The fungal infection was confined to the lungs in Cases 1 and 5. In Case 3 there were also small foci in the trachea, pharynx, and stomach, and in Case 4 early lesions were found in the meninges and brain (Table I). It appears that the fungus can reach the pulmonary alveoli in two ways: (1) By aspiration into the respiratory bronchioles and alveolar ducts with subsequent direct spread into the associated alveolar sacs and alveoli; (2) by colonizing the mucosa of the bronchi and then spreading through their walls. The examination of multiple blocks prepared from foci of different sizes enables one to reconstruct the evolution of the lesions and to study the effect of the fungus on various tissue components.

Alveolar Foci.—Under this heading we shall consider the lesions produced when the organism spreads into the lung parenchyma by the first of the two pathways mentioned. The macroscopic appearances of the early foci have already been described as small, often widely scattered, firm nodules which are deep red with yellowish centres. Microscopically each focus has a central area of

coagulation necrosis, appearing pink and granular in haematoxylin and eosin preparations. Fungal hyphae, staining bluish with haematoxylin, ramify in the necrotic debris. Cell outlines are lost but there may be numerous hyperchromatic nuclear fragments. Surrounding this central area there is a zone of consolidation. Here the alveoli are filled with fibrinous exudate ; the capillaries are dilated and the alveolar lining cells may be prominent and cuboidal. Sometimes a few fibroblasts are seen in the exudate, indicating early organization. This is confirmed by silver impregnation which reveals delicate reticulin fibrils amidst the exudate. Apart from a few lymphocytes, some macrophages and a very occasional plasmacyte, inflammatory cells are absent. At the periphery of the consolidated area there is a zone of intense vascular engorgement and intra-alveolar haemorrhage. Occasionally the alveolar exudate may be chiefly serous in character although this is rare and fibrin is usually abundant. Thus the typical early focus shows three zones: central necrosis, fibrinous consolidation, peripheral hyperaemia and haemorrhage (Figs. 11 and 12). As the fungus grows and thrusts its hyphae outwards, the zone of necrosis enlarges. The consolidated alveoli are overwhelmed, their cells are destroyed, their outlines become blurred and incorporated into the necrotic centre, whilst fresh fibrinous exudation occurs further out. As the necrosis extends into the consolidated alveoli the fibrin loses its fibrillary structure and characteristic staining reactions, becoming merged into the general granular debris. Thus special stains, such as Mallory's phosphotungstic acid haematoxylin or Lendrum's acid picro-Mallory method, consistently fail to reveal fibrin in the central zone. Although the necrosis usually effaces the alveolar outlines completely (in haematoxylin-and-eosin-stained sections) they may be preserved even throughout large lesions (Fig. 13). The fungus itself often shows degenerative changes such as swelling and fragmentation of the hyphae. This is most marked at the centre of the necrotic area where the mycelium tends to lose its affinity for haematoxylin and stains poorly in periodic-acid-Schiff preparations. Silver impregnation for reticulin and stains for elastic fibres show considerable preservation of these structures within the necrotic mass, although progressive fragmentation does tend to occur. Perls' reaction often reveals some haemosiderin at the periphery of the focus but little or none within the central zone. In summary, the lesion may be described as a centrifugally spreading coagulative necrosis, the advancing front being preceded by zones of fibrinous exudation, hyperaemia, and haemorrhage.

Bronchial Lesions. — Fungal mycelia grow within bronchial lumina and in this situation sporing heads may be seen (Fig. 14). There is a marked tendency for the aspergillus to spread through the bronchial walls into the adjacent lung. The earliest evidence of such penetration is an area of coagulation necrosis involving the epithelium and underlying connective tissue cells (Fig. 15). Such a segment appears granular and highly eosinophilic except in so far as there are scattered, deeply basophilic nuclear fragments. Beyond the limits of the necrosis the smooth muscle fibres are separated by oedema and the mucous glands are often greatly distended. The fungus then penetrates the dead tissue and extends outwards, a wave of necrosis preceding immediately before it. In this manner the whole thickness of the bronchial wall becomes necrotic and the hyphae reach the surrounding alveoli where they excite fibrinous exudation and haemorrhage as already described (Fig. 16). The alveoli undergo necrosis in their turn and so the lesion extends until a large volume of lung tissue may be involved. When passing through the bronchial wall, the fungus may grow outwards between adjacent cartilage plates, but cartilage itself is by no means immune to invasion by the hyphae (Fig. 17). When such invasion takes place, the cartilage matrix loses its basophilia and its metachromasia with thiazine dyes, so that it stains a uniform pink in haematoxylin and eosin preparations. The cells nearest the fungal filaments exhibit cytoplasmic swelling and vacuolation ; the nuclei undergo karyorrhexis or pycnosis. The cartilage plate shows progressive blurring of its outlines until it merges into the necrotic mass and can no longer be identified in haematoxylin and eosin sections. Silver impregnation usually reveals retention of a reticulin framework in the bronchial wall and the hyphae can be seen ramifying in the interstices of the argyrophilic mesh.

Vascular Lesions.—Invasion of blood vessels by aspergillus has been noted by several authors (McKee, 1950 ; Pesle and Monod, 1954 ; Oehlert and Düffel, 1958 ; Finegold *et al.*, 1959 ; Naji, 1959). We found involvement of small to medium-sized arteries and veins in all our cases. The smooth muscle and connective tissue cells of the vessel walls undergo coagulative necrosis and the fungus passes through into the lumen. The walls lose their normal structure and appear simply as eosinophilic rings, although the remains of elastic laminae can be demonstrated by suitable stains. Sometimes the junction between the affected and unaffected segments is remarkably sharp, and the

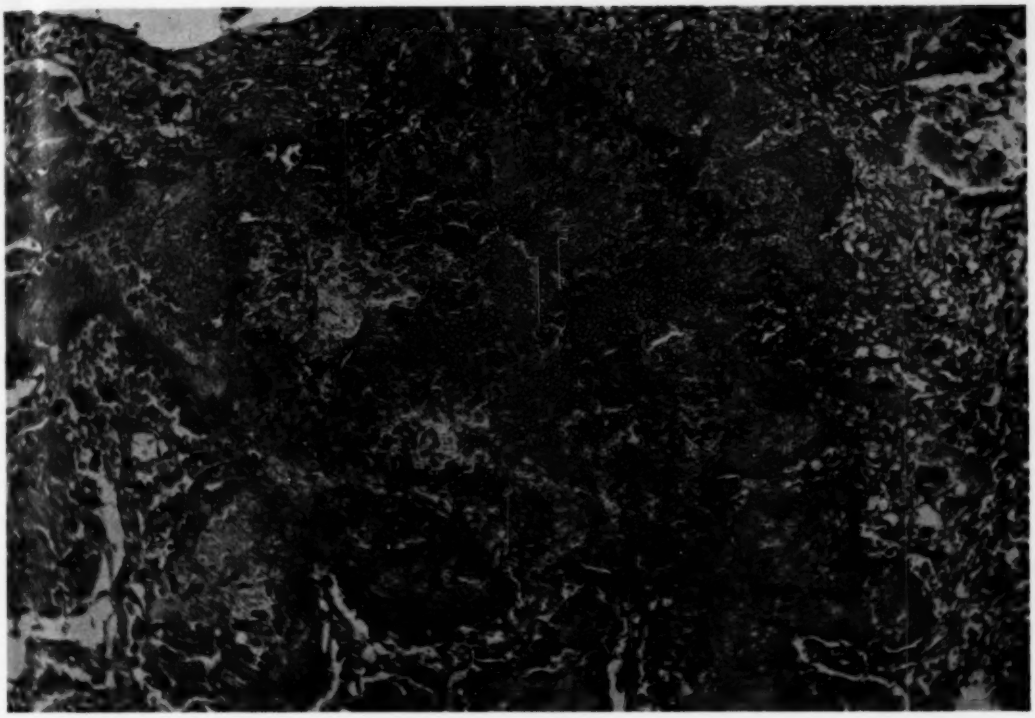

FIG. 11.—Case 4. Small alveolar focus showing fungus in necrotic centre and fibrinous exudate in surrounding alveoli. Haematoxylin and eosin ×150.

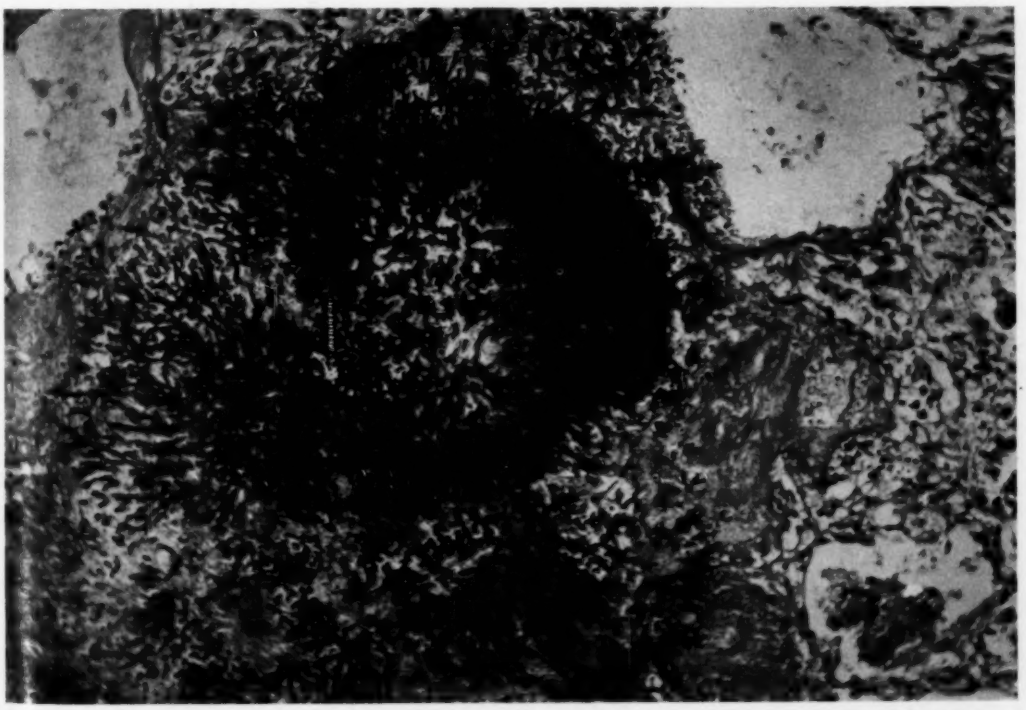

FIG. 12.—Case 4. Similar lesion to that illustrated in Fig. 11. Fungus coloured bright red in original section: black in photograph. Periodic acid-Schiff, haemalum ×150.

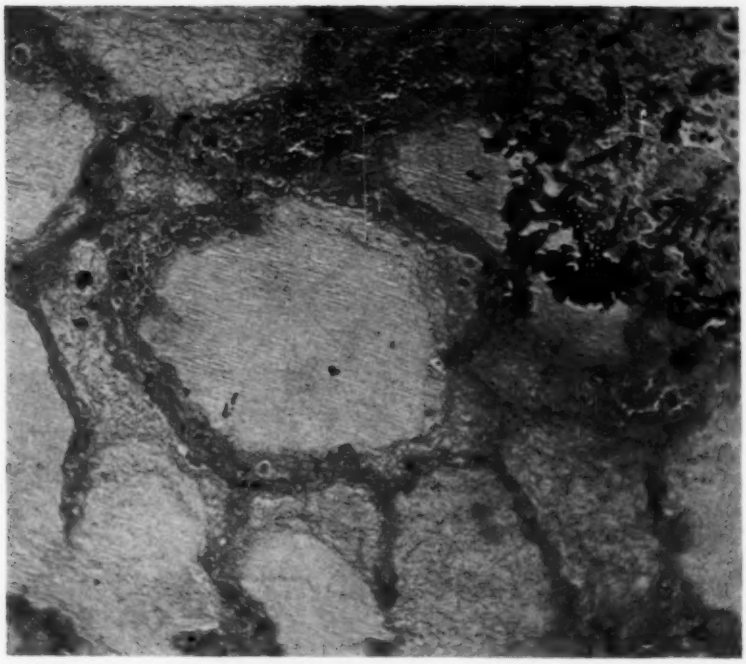

FIG. 13.—Case 1. Fungus growing in necrotic lung tissue. Alveolar outlines preserved. Haematoxylin and eosin ×160.

FIG. 14.—Case 1. Section through disorganized bronchus and adjacent necrotic lung tissue. Sporing heads are present in the lumen and hyphae have penetrated the bronchial wall. Haematoxylin and eosin ×150.

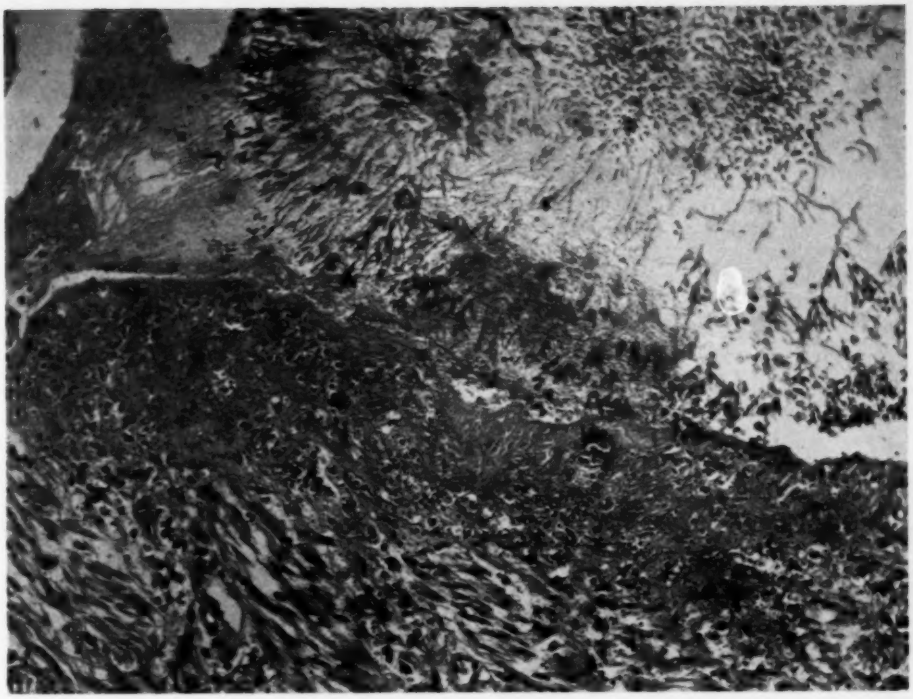

FIG. 15.—Case 4. Early bronchial lesion showing coagulation necrosis of mucosa and commencing invasion by fungus. Haematoxylin and eosin ×150.

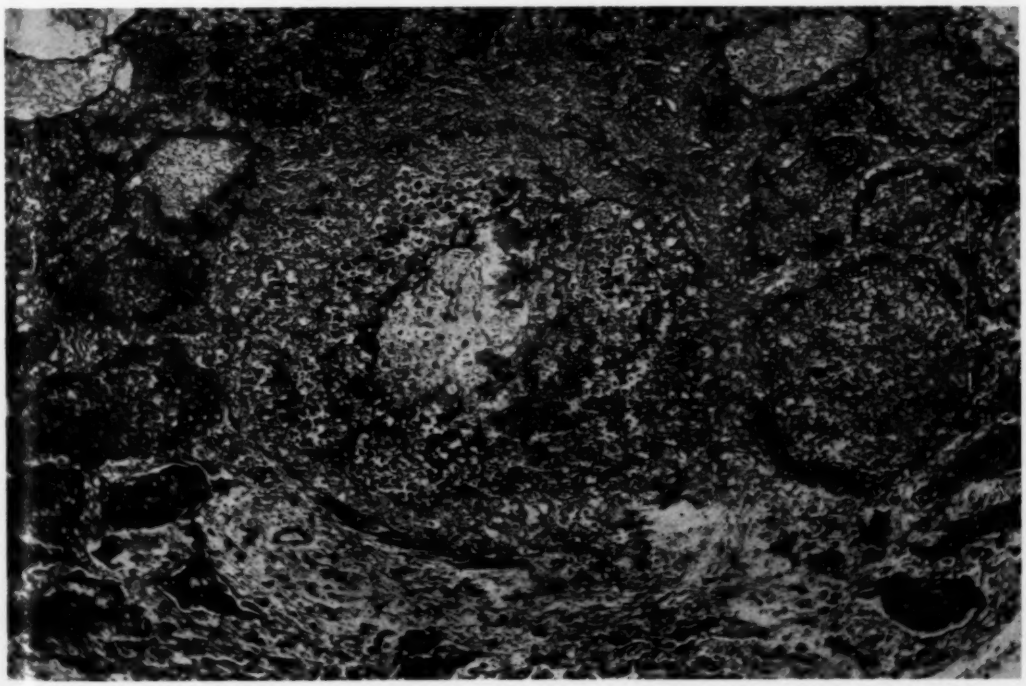

FIG. 16.—Case 1. Transverse section of a small bronchus showing hyphae in the lumen, complete disorganization of the wall, and abundant fibrinous exudate in surrounding alveoli. Fibrin coloured deep blue in original preparation: black in photograph. Phosphotungstic acid-haematoxylin ×75.

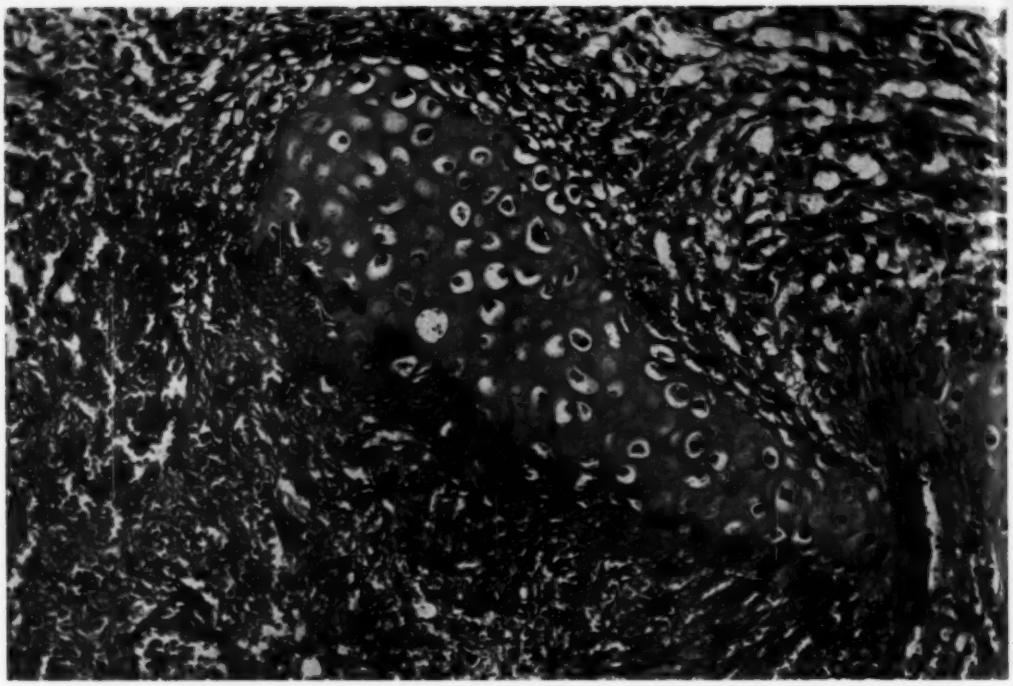

FIG. 17.—Case 4. Invasion and partial destruction of bronchial cartilage by fungus. Haematoxylin and eosin ×216.

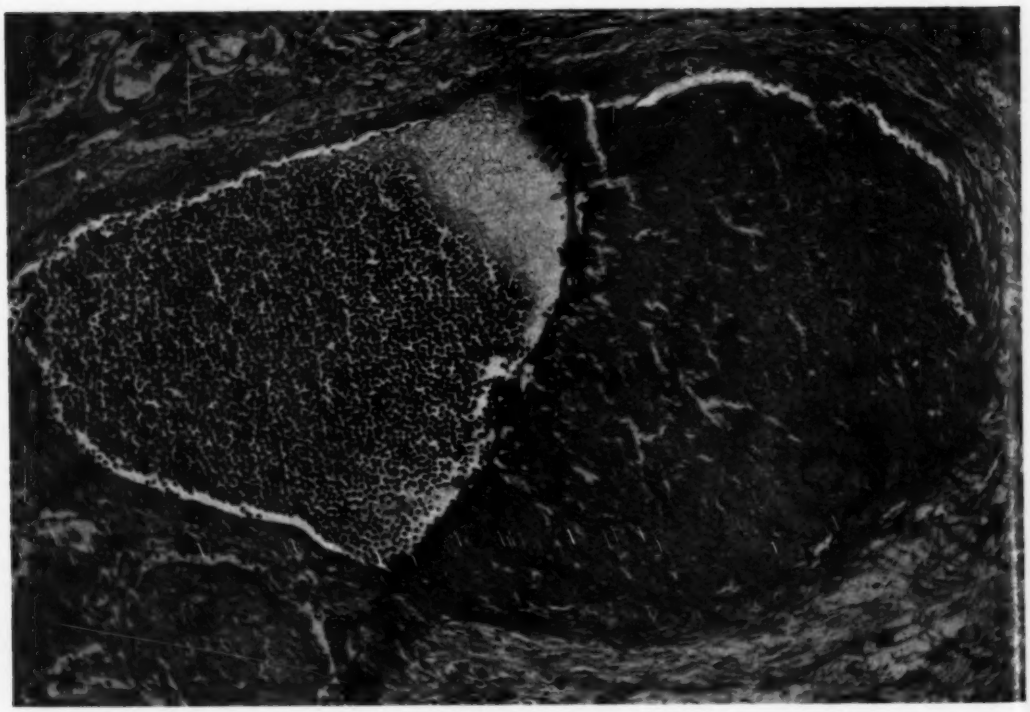

FIG. 18.—Case 3. Pulmonary blood vessel partly destroyed and occluded by fungus. Note leukaemic cells in vessel lumen and the exudate in the alveoli. Haematoxylin and eosin ×75.

way in which the mycelium engulfs the vessel, destroying the tissues but leaving vague structural outlines in its wake, is reminiscent of the earth's shadow crossing the moon during a lunar eclipse. Such an "eclipsing" lesion is shown in Fig. 18. Within the lumina the hyphae often lose their basophilia and stain pink with eosin, contrasting with those portions of the fungus that are still extravascular. Whether this change of staining property is due to the deposition of eosinophilic material upon the hyphae or to some other cause is uncertain. Suitable staining procedures sometimes show a halo of radiating fibrin filaments around the hyphae (Fig. 19). However, we wish to stress that the material occluding the vascular lumina in our cases consists of fungus and an eosinophilic granular precipitate containing very little fibrin. So that, although other workers refer to "thrombosis" of vessels invaded by aspergillus, the occlusive lesions seen in our specimens have neither the morphology nor the tinctorial properties of ordinary fibrin thrombi. Vascular obstruction may be an important factor in the pathogenesis of the necrosis which characterizes the fungal invasion, but a study of small lesions suggests that toxic products may also cause tissue death by direct action. Massive lesions such as those seen in the lungs of Case 1 and the kidney of Case 2 probably result from a combination of both factors, ischaemic and toxic. Hinson *et al.* (1952) refer to the invasion of infarcts by aspergillus and speak of such lesions as saprophytic. While this may well occur, it seems certain that the fungus itself is able to produce large infarct-like areas of necrosis, especially in the lungs (Fig. 13).

Gastric Lesions.—Sections of the ulcers from Case 3 show a non-suppurative tissue reaction basically similar to that already described for the lungs. Within the lesions, the mucosa has undergone coagulation necrosis to form an eosinophilic mass devoid of nuclei but still retaining vague tubular outlines. The fungus is growing in the necrotic tissue and has penetrated into the submucosa where many blood vessels have been invaded. Vascular dilatation, sero-fibrinous exudation, and red cell extravasation are prominent in the submucous layer (Fig. 19).

Cerebral Lesions.—In Case 4 there is focal involvement of the leptomeninges, the fungus being present within the lumina of small blood vessels. The vessels are dilated and there is a sero-fibrinous exudate in the subarachnoid space. Inflammatory cells are very scanty. Hyphae have penetrated the vessel walls and there is early invasion of the brain substance. Small haemorrhages are present in the cortex several millimetres beyond the limits of the fungal invasion.

FIG. 19.—Case 3. Section showing fungus within a blood vessel of the gastric mucosa. Note the small amount of fibrin deposition around the hyphae. The adjacent connective tissue is oedematous and contains a fibrinous exudate. Fibrin coloured deep blue: hyphae, tan. Phosphotungstic acid-haematoxylin ×150.

Suppurative Lesions.—The pulmonary lesions of Case 2 comprise numerous small, widely scattered foci of bronchopneumonia (Fig. 20). The alveolar ducts and associated alveoli are filled with an exudate rich in polymorphs amongst which swollen fungal hyphae can be identified. The lesions in the myocardium, thyroid, spleen, kidneys, and liver have the usual features of suppurative inflammation and need not be described further (Figs. 21 and 22). The pericardial exudate is composed mainly of a thick layer of fibrin, but small foci of suppuration are also present (Fig. 23). Invasion of blood vessels, with acute arteritis, is seen in several sites, but is especially prominent in the right kidney where large vessels are involved (Fig. 24). This has resulted in extensive infarction affecting the upper half of the organ.

Discussion

The following aspects will be considered: (1) The classification of aspergillosis and the distribution of the lesions; (2) factors influencing the

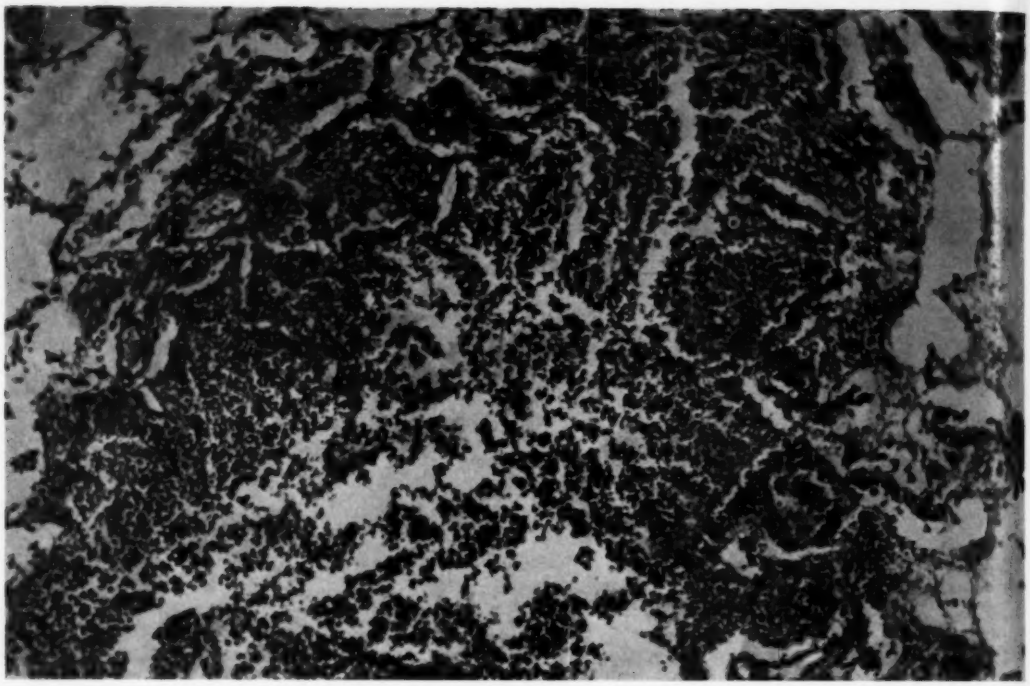

FIG. 20.—Case 2. Section of small suppurative focus in lung. Hyphae could be seen on high-power examination. Haematoxylin and eosin ×150.

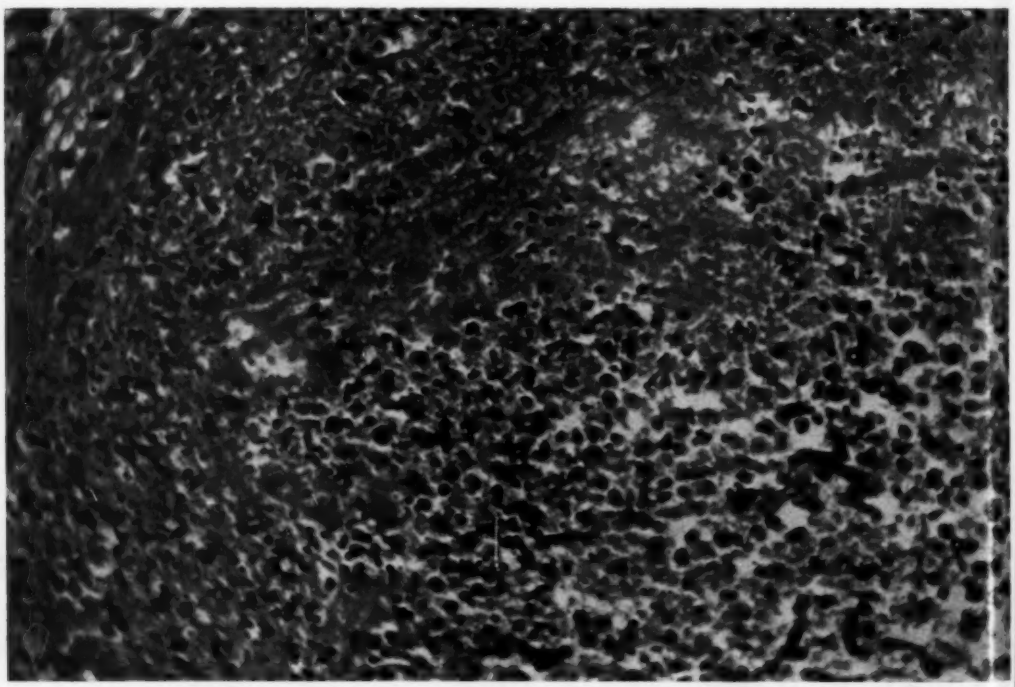

FIG. 21.—Case 2. Section of splenic abscess showing fragmented mycelium in pus. Haematoxylin and eosin ×430.

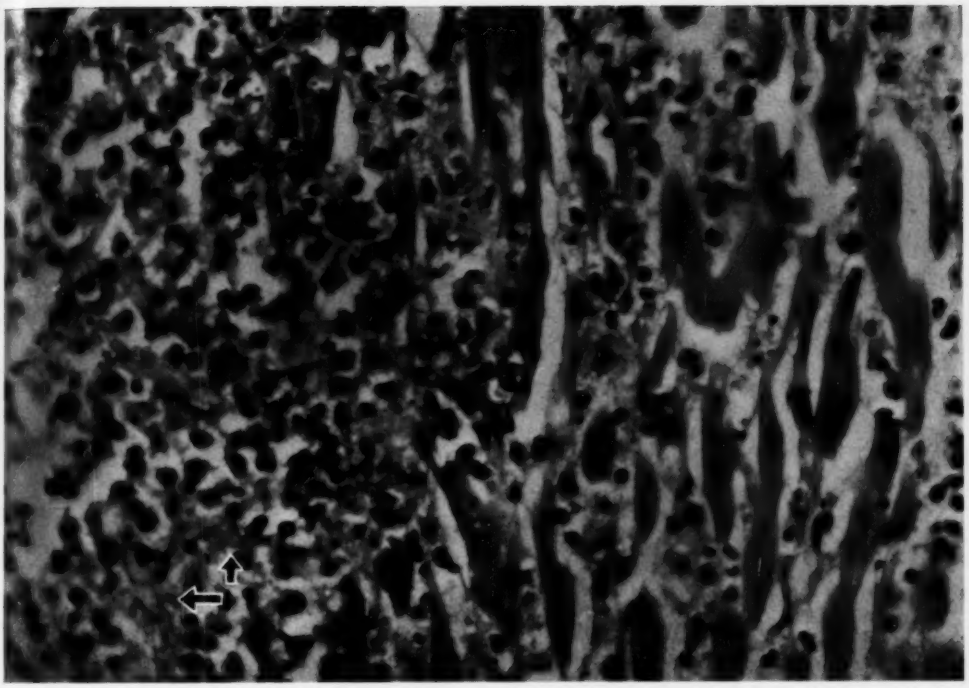

FIG. 22.—Case 2. Section of myocardial abscess showing pus cells and swollen hyphae. Haematoxylin and eosin ×520.

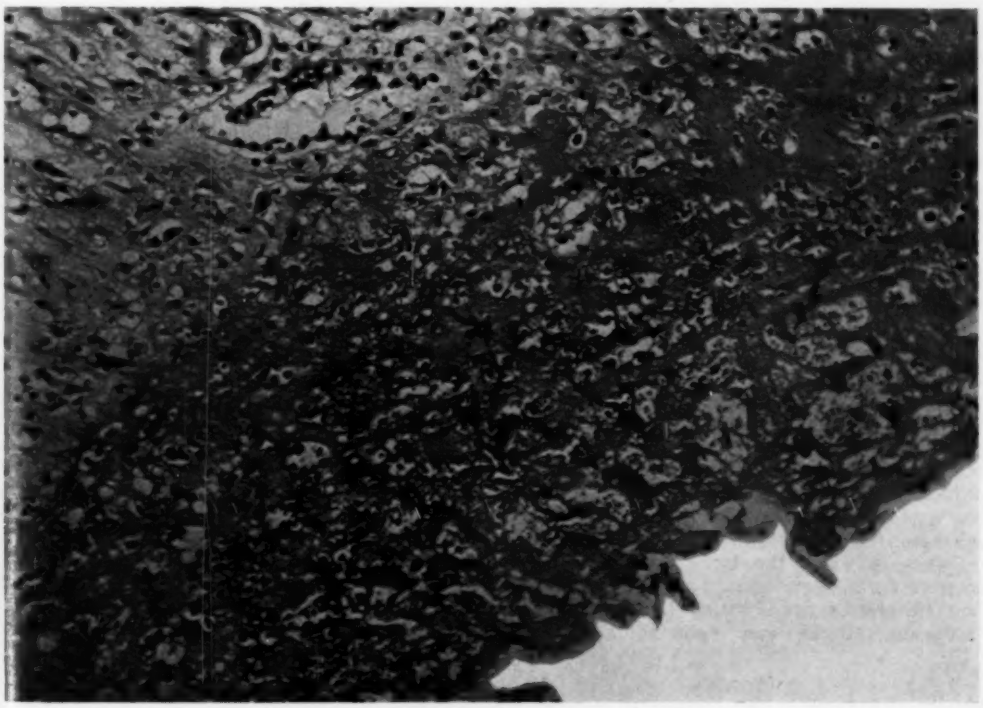

FIG. 23.—Case 2. Section of epicardium showing hyphae in fibrinous exudate. Haematoxylin and eosin ×216.

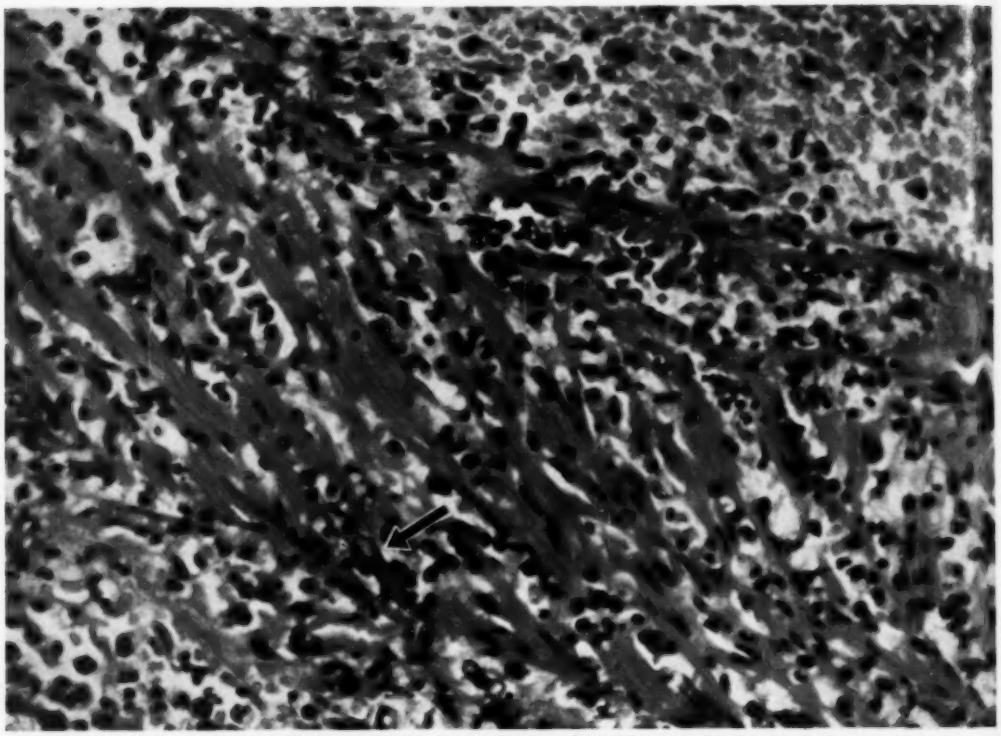

FIG. 24.—Case 2. Section from the wall of a large renal blood vessel showing oedema and polymorph infiltration of the media and the presence of swollen hyphae. Haematoxylin and eosin ×430.

invasion of the body by the fungus; (3) factors influencing the nature of the tissue reactions.

Classification and Distribution of Aspergillus Lesions.—Various classifications of aspergillosis have been suggested. These are based on (*a*) whether the disease is secondary or primary, i.e., whether a predisposing disease is or is not present; (*b*) the extent of spread (localized or disseminated); (*c*) the pathology of the lesion; and (*d*) the presence or absence of allergy (Hinson *et al.*, 1952; Hinson, 1958; Finegold *et al.*, 1959). With regard to pulmonary disease, there are several well-characterized types: (1) Aspergillus bronchitis in which the fungus grows within the bronchial lumina and the mucosa shows only superficial changes (Körtge, 1952; Segretain, 1958); (2) allergic, in which the bronchi contain fungus, mucus, fibrin, eosinophils, Curschmann's spirals, and Charcot-Leyden crystals. The patients suffer asthmatic attacks and eosinophilia may be present in the peripheral blood (Hinson *et al.*, 1952); (3) "mycetoma" or "intracavitary aspergilloma." This comprises a mycelial mass (fungus ball), usually reddish-brown, lying in a saccular dilatation of a bronchus. The bronchial epithelium is often intact and the adjacent connective tissues are infiltrated with plasmacytes, macrophages, and polymorphs in varying numbers (Monod, Pesle, and Segretain, 1951; Pesle and Monod, 1954; Hinson, 1958; Naji, 1959); (4) invasive, in which the fungus spreads beyond the confines of the bronchi into the alveolar parenchyma of the lung (Lapham, 1926; Penta, 1955; Finegold *et al.*, 1959; Naji, 1959).

Spread from the lungs by the blood stream may produce fungal lesions in other parts of the body such as the brain, meninges, endocardium, myocardium, pericardium, kidneys, liver, spleen, thyroid, lymph nodes, pancreas, and bones (see review by Grcevic and Matthews, 1959). Dissemination to the brain and meninges was present

in our Case 4. In Case 2 fungal lesions were present in the pericardium, myocardium, thyroid, spleen, kidneys, and liver. Although the respiratory tract is the usual portal of entry, the fungus can also colonize the mucosa of the alimentary tract (Welsh and McClinton, 1954; Zimmerman, 1955; our Case 3). It is also possible that these organisms may occasionally be introduced by therapeutic injections. Wybel (1952) reported a case of mycotic granuloma of the leptomeninges in a patient who had received intrathecal injections of penicillin for pneumococcal meningitis three years before her death. The clinical course of the granuloma simulated a spinal cord tumour.

Factors Influencing Invasion of the Body by the Fungus. — The importance of environmental (occupational) factors and of pulmonary lesions has already been mentioned. Apart from this there is evidence that tissue invasion by fungi is facilitated by the administration of various antibiotics, chemotherapeutic drugs, and hormones. Keye and Magee (1956), reviewing the incidence of fungal infections amongst 15,845 consecutive necropsies performed from 1919 to 1955, found a conspicuous increase in the number of secondary fungous infections since 1947. Histoplasmosis, cryptococcosis, mucormycosis, candidiasis, and aspergillosis had all increased. They also noted a greater tendency to systemic dissemination of the fungi during recent years. An especially high incidence was observed in cases of leukaemia and malignant lymphoma. These authors believed that the rising frequency was related to the administration of multiple antibiotics, chemotherapeutic compounds, and cortisone. Craig and Farber (1953) observed 13 examples of disseminated visceral fungal infections amongst 175 cases of acute leukaemia in children who had come to necropsy since the introduction of anti-folic-acid compounds, triethylmelamine, cortisone, and A.C.T.H. There were 11 infections with candida and one each with mucor and cryptococcus. No case of disseminated visceral mycosis was found in untreated leukaemic children. Zimmerman (1955) also reviewed the rising incidence of fungal infections complicating other diseases. He concluded that both aspergillosis and candidiasis were increasing as a result of treatment by antibiotics, chemotherapeutic agents, and nitrogen mustards. Torack (1957) reported 13 cases of fungus infection (moniliasis, mucormycosis, and aspergillosis) associated with antibiotic and steroid therapy. His cases fell into two categories. In those patients receiving antibiotics alone, the fungi showed only surface proliferation; in cases where both antibiotics and cortisone had been given, the fungi actively invaded the tissues. Sidransky and Friedman (1958) saw 15 examples of secondary fungal infection amongst routine necropsies from 1955 to the end of 1957. There were five cases of aspergillosis; each of these had received treatment with cortisone and "broad-spectrum" antibiotics. *Aspergillus flavus* was cultivated from three cases. Finegold *et al.* (1959) reported 12 cases of aspergillosis. Seven were suffering from carcinoma, leukaemia, or Hodgkin's disease. Six had been given steroids. Four had received other "anti-neoplastic" therapy including irradiation to the spleen, "myleran," "leukeran," and "amethopterin."

These clinical observations are supported by experimental studies. Huppert, MacPherson, and Cazin (1953) found that aureomycin caused a significant growth stimulation of *Candida albicans* in broth cultures. Seligman (1953) demonstrated enhanced virulence of *Candida albicans* by terramycin, aureomycin, and cortisone in experimental animals. Mankowski and Littleton (1954) found that the administration of cortisone reduced the survival times of mice infected with *Candida albicans, Aspergillus sp., Penicillium sp., Histoplasma capsulatum,* or *Cryptococcus neoformans.* Sidransky and Friedman (1959) exposed mice to aerosols containing dry, viable spores of *Aspergillus flavus.* Untreated animals and those given only antibiotics developed a transient pneumonitis. In those mice receiving cortisone, or cortisone and antibiotics, there was rapid germination of the spores and spread of hyphae through the lungs. The authors comment that the histological appearances in the lungs of the cortisone-treated mice closely resembled those found *post mortem* in human cases of pulmonary aspergillosis.

It therefore seems likely that the establishment and dissemination of aspergillus infection are dependent on a number of factors: (1) A local lesion such as bronchiectasis or a tuberculous cavity; (2) a high concentration of spores in the environment (occupational hazards); (3) depression of antibody and cellular defence mechanisms by generalized disease, malignant lymphomas and leukaemia being of paramount importance in this respect; (4) further depression of defence mechanisms by radiotherapy, antimetabolites, radiomimetic drugs, steroids, or A.C.T.H.; and (5) disturbance of the "normal" respiratory flora by the administration of antibiotics (Tomaszewski, 1951). We consider factors (3) and (4) to be those of greatest significance in our own cases.

Certain Factors Influencing Nature of Tissue Reactions.—Baker (1947) and Symmers (1958) have described the various tissue reactions that may be found in fungal infections. The pattern varies with the type of fungus, its location, the duration of the lesion, the presence or absence of allergy, and the general condition of the host. According to the circumstances one or more of the following may be seen: intracellular parasitism, suppuration, chronic non-specific inflammatory changes, fibrosis, tuberculoid reactions, caseation, "foreign-body" reactions, and "no reaction." The changes most often seen in the invasive varieties of aspergillosis are necrosis, acute inflammation, and suppuration. It will be recalled that four of our cases displayed foci of coagulation necrosis associated with exudation and haemorrhage but with a virtual absence of inflammatory cells. Necrosis and haemorrhage have been reported by other workers such as Heppleston and Gloyne (1949), Rankin (1953), Naji (1959), and Grcevic and Matthews (1959). Others have described granulomatous lesions with epithelioid cells and giant cells. Such granulomas have been observed both in humans (Cawley, 1947; Iyer, Dodge, and Adams, 1952; Finegold *et al.*, 1959) and experimental animals (Henrici, 1940; Cooper, 1946; Oehlert and Düffel, 1958). Henrici (1940) studied the lesions produced in rabbits by injecting aspergillus spores. He found that massive doses produced foci of necrosis and haemorrhage. Moderate doses led to abscess formation, while small numbers of spores resulted in "tubercles" with central caseation. In lesions of the last type, the so-called actinomycetoid form of the fungus was sometimes found. This consists of short filaments of thin mycelium radiating from a portion of normal-sized mycelium; the ends of the filaments are often capped by acidophilic material. Actinomycetoid bodies have been observed in human lesions (Oehlert and Düffel, 1958), but they were not present in our own cases. Henrici (1940) believed the epithelioid-cell granulomas and the actinomycetoid forms to be manifestations of hypersensitivity. If this is so, their absence from our cases is not surprising in view of the frequent poverty of immunological mechanisms in Hodgkin's disease (Dubin, 1947) and their impairment in leukaemia following treatment with steroids (Larson and Tomlinson, 1953). Grcevic and Matthews (1959) have also discussed the possibility of hypersensitivity reactions in patients with aspergillosis. They reported three cases of disseminated infection and described certain features which they considered indicative of hypersensitivity. The features they stressed were diffuse joint pains, symmetrical facial rash, "onion peel" arteriolar changes in the spleen, and erythrophagocytosis in lymph nodes. The lesions in their cases were acute and suppurative: no giant-cell granulomas were observed.

There is no evidence that the fungus can produce a true exotoxin, although certain toxic antibiotic and tumour-inhibiting substances have been isolated from cultures of *Aspergillus fumigatus* (Soltys, 1944; Asheshov and Strelitz, 1945; Petermann, Hamilton, and Reilly, 1952). Oehlert and Düffel (1958) found anisotropic crystalloid bodies within fungal networks lining the bronchi of their patient. They suggested that these crystals were toxic metabolites of the fungus. Henrici (1939) extracted a "cell sap" rich in endotoxin from *Aspergillus fumigatus* and *Aspergillus flavus*. Subcutaneous injection of the cell sap into rabbits and guinea-pigs resulted in necrosis, oedema, and polymorph infiltration. Intravenous injections produced pulmonary haemorrhages, renal tubular necrosis and lobular hepatic necrosis in rabbits; in guinea-pigs there were nervous symptoms leading to paralysis. The sap was also haemolytic to sheep's erythrocytes. Such toxic agents, liberated from degenerating fungal filaments and diffusing into the tisues surrounding a mycelial mass, could produce the type of tissue response observed in our cases. Tissue damage occurring in this manner may well pave the way for further fungal invasion and the centrifugal spread of the lesion (Riddell, 1958). In four of our cases the depression of antibody and cellular defences apparently allowed the rapid development of the lesions in a "pure" state and it was possible to study the effect of the toxic substances on different tissue components without the complicating factor of an inflammatory-cell infiltrate. The diffusable toxic products can cause necrosis of epithelial cells, connective-tissue cells, and muscle fibres. They affect the ground substance of cartilage which loses its basophilia and is finally destroyed together with the chondrocytes. Reticulin, elastic fibres, and collagen are more resistant and can often be demonstrated deep in necrotic zones. Damage to blood vessels results in exudation and the extravasation of erythrocytes, while more severe effects are manifest by necrosis of the vessel walls and occlusion of their lumina. The vascular obstruction will then lead to further tissue degeneration and necrosis. We have already stressed the small amount of fibrin that could be demonstrated in the material occluding the vessels of our cases, and have described the dissolution of fibrin in the alveolar exudate as it becomes invaded by the fungus. These observations suggest a fibrino-

lytic action of the diffusable products, and it is interesting to note that a substance having such activity has recently been isolated from *Aspergillus oryzae* (Stefanini, Adamis, Soardi, Horace, Marin, and Mele, 1959). On the other hand the hyphae in the pericardial exudate of Case 2 do not appear to have had any significant lytic effect on the fibrin in their vicinity, although digestion has occurred in the suppurative foci. It is possible that the large surface area of the serous membranes allows such an abundant exudation that the fibrin remains constantly in excess of any fibrinolytic substance diffusing from the fungus. The virtual absence of neutrophils from the peripheral blood during the last days of life probably accounts for the absence of suppuration in Cases 1, 3, 4, and 5. This interpretation accords with the findings of Torre and Leikin (1959). Using the Rebuck skin window test these authors demonstrated a poor inflammatory response in leukaemic patients who had significant neutropenia. However, it is interesting to recall that in our Case 1, staphylococcal abscesses were present in the lungs, so that a suppurative type of inflammatory reaction was still possible; this indicates the poor pyogenic properties of aspergillus relative to the staphylococcus under identical environmental conditions. The only patient who did not have a severe terminal neutropenia (Case 2) developed widespread suppurative lesions as already described. It is possible that the toxic substances may be absorbed into the blood stream and exert an effect on distant parts of the body. Chortis (1952) recorded his clinical impression that pulmonary aspergillosis can cause toxic effects on the myocardium and central nervous system, although he produced no pathological evidence for this statement. Oehlert and Düffel (1958) found necrosis and calcification of the renal tubules in rats that had been given intraperitoneal injections of *Aspergillus fumigatus*. They also described fibrinoid swelling of the walls of small and medium-sized blood vessels in the livers, kidneys, and hearts of the animals. In our own cases we have been unable to find changes in distant organs that could be attributed to toxic metabolites of the aspergillus. It may, of course, be extremely difficult to distinguish between lesions produced this way and those resulting from other factors such as anaemia, cardiac failure, and therapeutic agents.

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THE DIFFERENTIAL DIAGNOSIS OF TUMOUR CELLS IN CIRCULATING BLOOD

BY

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White-cell concentrates were made by a dextran sedimentation technique from the blood of 140 cases of malignant disease and 60 controls. The slides were searched for tumour cells and for other unusual cells with which they might be confused. Several million white cells were scanned in the slides from each case.

Acceptable tumour cells were identified in the blood of seven patients, none of whom survived for more than a few months. These cells are illustrated, as well as various other cell types which may have been confused with malignant cells in the past. It is concluded that the cytology of white cell concentrates should be further explored before statistics about the occurrence of circulating tumour cells are accepted.

Metastasis of malignant tumours by way of the blood stream is common, but until the last five years there were very few descriptions of the isolation of tumour cells from the circulating blood (Marcus, 1919; Jonescu, 1931; Pool and Dunlop, 1934). It was usually thought that metastases were thrown off intermittently and in small numbers so that there was little likelihood of finding them in a random blood sample.

An exception is provided by the malignant conditions of the haemopoietic system—leukaemia, reticulosarcoma, multiple myeloma—which will not be considered here. Another is neuroblastoma, which occasionally mimics an acute leukaemia (Undritz, 1948, and in the Sandoz atlas; Christenson, Ultmann, and Mohos, 1956; Okinaka, Nakao, Kinugasa, Takaku, and Ohtsuki, 1956).

With the introduction of effective methods of separating the red and white cells, new attempts have been made to identify circulating cancer cells. Reports have now come from various centres claiming to have found such cells in a surprisingly high proportion of cases of malignant disease, ranging from 16 to 59% (Engell, 1955, 1959; Fisher and Turnbull, 1955; Moore, Sandberg, and Schubarg, 1957; Roberts, Watne, McGrath, McGrew, and Cole, 1958; Pruitt, Hilberg, and Kaiser, 1958; Fletcher and Stewart, 1959; Reiss, 1959).

After practically a century of morphological haematology it might be thought that all the types of cell occurring in the blood would already be well known. It turns out, however, that this is not the case, and the new techniques of white cell concentration are beginning to reveal a remarkable variety of non-malignant cells which are seldom or never figured in atlases of the blood. Unfortunately the statistics of the occurrence of supposedly malignant cells in the blood have been compiled and published before, and not after, a thorough study of the other lesser-known cells which might cause confusion, and it seems very likely that malignant cells have been overdiagnosed for this reason.

Several of the authors recommend techniques which have not been extensively used in the past for the study of blood or bone marrow. The Papanicolaou method, for instance, does not seem to us to have any advantage in this field over the Romanowsky stains which are universally used in haematological diagnosis.

We hope in this paper to help to remedy the position by illustrating a variety of different non-malignant cells, some of which are of known origin and others unknown, as well as some which we consider to be acceptable tumour cells. The reader is also referred to Sandberg, Moore, and Schubarg (1959) for illustrations of certain non-malignant cells found in the blood.

Material and Technique

Two hundred and fifty blood samples were examined from 200 persons. One hundred and forty of these were cases of malignant disease, mostly advanced; and 60 were either normal or suffered from other diseases. A particular effort was made to find patients in whom abnormal cells might be expected, for instance, those with leuco-erythroblastic anaemia and with terminal illnesses. Most of the samples were from the antecubital vein, but 45 samples were taken in the operating theatre from veins draining either malignant tumours or other "control" lesions.

The method used was similar to that of Sandberg and Moore, but we found that the use of dextran instead of fibrinogen solution avoided the formation of lumps of fibrin with adherent white cells or platelets.

Blood, 4 ml., was mixed with 1 ml. of 6% dextran solution in 0.9% saline, containing 45 units of heparin per ml., in a siliconed tube. This was left undisturbed for 20 to 30 min., by which time the red cells had sedimented. The supernatant fluid was transferred to a siliconed conical centrifuge tube and centrifuged at less than 1,000 r.p.m. for 10 minutes. The whole of the deposit was then spread on a series of slides by the method used for making blood films. Usually about twelve films were made. All of these were fixed in methyl alcohol, stained with May–Grünwald–Giemsa, and mounted in DPX. The whole of every slide was examined under the 16 mm. objective and unusual cells marked and studied under higher powers. By this means an adequate view was obtained of several millions of white cells from each specimen. These cells had suffered no visible alteration from the normal appearances as seen in directly made blood films.

Although the scanning of these films is very laborious, losses of possible tumour cells are minimized. Any further concentration procedures involve not only loss of cells, but deterioration of the cells which remain.

Non-malignant Nucleated Cells Occurring in White Cell Concentrates

Granular Series.—These are the predominant cells in white cell concentrates and need not be described here. Occasionally myelocytes show defective granulation and, being large, attract attention. Myeloblasts are usually too small to be confused with tumour cells.

Lymphocytes.—Next in frequency to the neutrophil granulocytes, the lymphocytes can hardly be confused with neoplastic cells. A very occasional large form may occur, but rarely reaches the size of the smallest oat cell.

Plasma Cells.—Cells of the plasmacyte series are probably always present. They are familiar to haematologists as "Türk cells." In direct blood films from normal people only an occasional Türk cell can be found, but they are prominent in white cell concentrates owing to their deep cytoplasmic basophilia. No full description need be given, as there are excellent pictures in the haematological atlases (Undritz, 1952; Osgood and Ashworth, 1937; Moeschlin, 1947; Bessis, 1956).

Türk cells are very variable, both in size and in nuclear chromatin pattern. Some forms have a fine chromatin pattern with distinct nucleoli, and as the nuclear diameter may reach 16 μ or occasionally even more, they can cause confusion to the inexperienced. The cytoplasm invariably shows deep basophilic staining, a feature which is hardly ever found in free tumour cells, so that this is an important differential point (Figs. 1 and 2). They also sometimes show cytoplasmic hyaline droplets (Mott cells or grape cells).

Türk cells have been found in mitosis in many of the white cell concentrates; in fact it is rare to see division occurring in any other type of cell, e.g., normoblasts, myelocytes, megakaryocytes. The description and illustrations given by Nowell (1960) of dividing cells in cultures from peripheral blood are reminiscent of the same cell type.

Monocytes.—Monocytes are always abundant in white cell concentrates and although there are occasional large forms possessing nucleoli they have not in our experience caused difficulties of identification.

Macrophages.—This name has been applied to monocytes which develop vacuoles and evidence of phagocytosis, and are found in the blood in septicaemia. Cells similar to the alveolar macrophages of the lung have been recorded by Sandberg *et al.* (1959). We have seen isolated examples of macrophages in blood from a variety of cases, and occasionally their contents have been of a specific kind; for instance, in a case of Gaucher's disease (Fig. 3) these cells contained the characteristic fibrous or crystalline material, and in a case of malignant melanoma with melanosis there were macrophages containing melanin (Goodall, Spriggs, and Wells, 1960).

Megakaryocytes.—The bare nuclei of megakaryocytes are normally present in the blood, but in very small numbers. Using 4 ml. volumes we have found a few in all normal samples examined, and their numbers are often increased in disease.

Megakaryocyte nuclei are often seen attached to platelet agglutinates, as shown in Fig. 156 of the Sandoz Atlas of Haematology. They also occur separately, and may be of considerable size with several lobes (Fig. 4). In Papanicolaou preparations they appear markedly hyperchromatic (Fig. 5).

Examples with cytoplasm also occur, but in our experience these have only been found in disease,

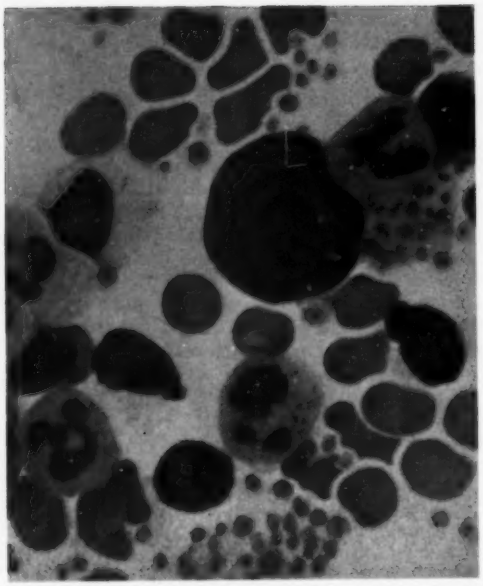

FIG. 1.—"Türk cell" or proplasmacyte. These cells are normally present in the blood, and are remarkable for their deeply basophilic cytoplasm. The example shown has several visible nucleoli. Case of cerebral tumour (×1,300).

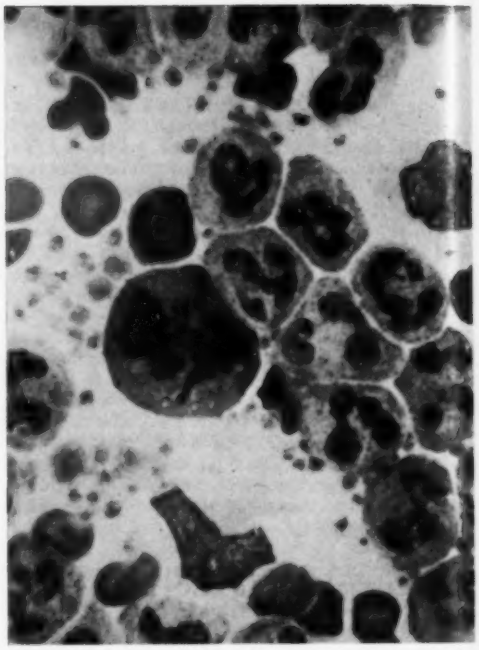

FIG. 2.—"Türk cell" in mitosis. This is seen in many cases where the plasma cell series are numerous. Same case as Fig. 1 (×1,300).

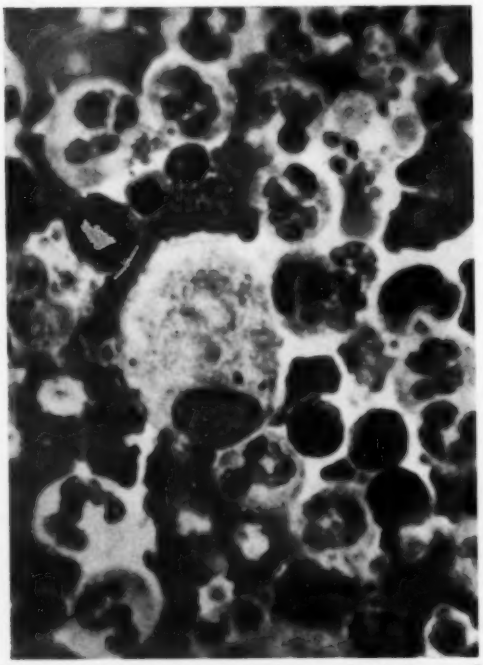

FIG. 3.—Lipid-containing macrophage in venous blood of a case of Gaucher's disease (×1,300).

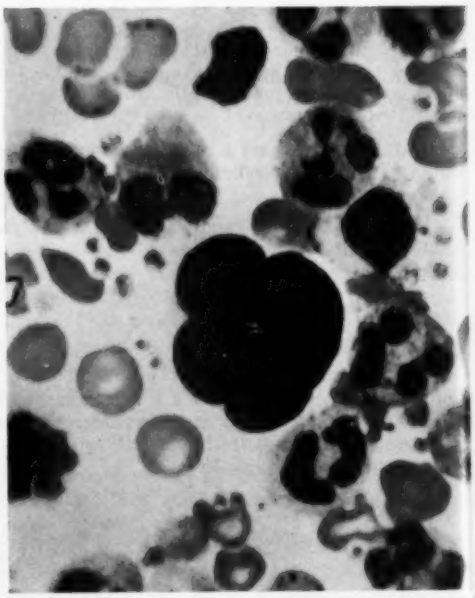

FIG. 4.—Megakaryocyte nucleus in peripheral blood. These have been found in nearly every sample so far examined, including all those taken from healthy subjects. This one is from a case of carcinoma of the pancreas (×1,300).

for example, in malignant disease and in leuco-erythroblastic anaemia (Figs. 6 to 12). Di Guglielmo (1923) illustrated them in chronic myeloid leukaemia. Their morphology is the same as in bone marrow and very large ones are occasionally seen, for instance, we have observed one measuring 50 μ across. In one patient with a leuco-erythroblastic anaemia due to myelosclerosis we found one in mitosis (Fig. 10). Sometimes there is a high nucleo-cytoplasmic ratio, and instead of the usual profuse, pale, violet-stained cytoplasm with eosinophilic granules there is a narrow rim of cytoplasm showing intense basophilia (Figs. 7, 8, and 11). These correspond to the megakaryoblasts illustrated by Bessis (1956) and such cells may well have been confused in the past with tumour cells. It should be emphasized again that deeply basophilic cytoplasm is very uncommon in malignant cells.

Osteoclasts.—Osteoclasts are figured in the Sandoz atlas and by Tischendorf and Heckner (1950).

We have only on one occasion seen a probable osteoclast in the circulating blood (Fig. 13). The cell measured 33 $\mu \times 51$ μ and contained nine oval nuclei, several of which had distinct nucleoli. The specimen was taken from the pulmonary vein of a patient with carcinoma of the lung, about 10 minutes after rib resection.

Endothelial Cells.—Cells from the vascular endothelium are uncommon in white cell concentrates, but when they occur they can cause difficulty in identification. It is useful to make imprints of veins, arteries, and the chambers of the heart in order to study these cells, which show surprising variations such as forms with multiple or giant nuclei.

Typically, endothelial cells are found in sheets, and these have ragged edges with peripheral cells coming loose. Even when free from each other several are usually found close together (Fig. 14). The nuclei are oval, and a typical measurement would be 15 $\mu \times 9$ μ; the mean diameter of a single nucleus varies from 10 μ to 14 μ with rare exceptions above and below. The chromatin is rather denser than that of a monocyte, and there is often a small visible nucleolus. The cytoplasm is profuse and gives the impression of being torn and ragged at the edges. It stains pale blue and has a cloudy texture. Sometimes these cells are seen with pyknotic nuclei, or else with pink staining structureless nuclei which are obviously dead. A multinucleate form with pyknotic nuclei is shown in Fig. 15.

Tissue Mast Cells.—These are rarely seen in concentrates of circulating blood. They are exactly as described occurring in the bone marrow (Sandoz atlas), and one of them is shown in Fig. 16.

Erythropoietic Cells.—In three cases we have found in antecubital venous blood taken during operation small clumps of erythropoietic cells regarded as probable marrow emboli, since all three cases had had a rib resected from five to 10 minutes previously. In two cases there were 14 and 16 normoblasts respectively, and a central macrophage or reticulum cell (Fig. 17). The other case had four components lying near together in the same streak when the film was spread, with a total of 27 normoblasts, each fragment associated with a macrophage or a reticulum cell and one with an early megakaryocyte.

Trophoblastic Cells.—Douglas, Thomas, Carr, Cullen, and Morris (1959) have found mutlinucleate cells in blood from the broad ligament veins of pregnant women, especially early in pregnancy. The larger of these cells are very distinctive, and are believed to be wandering trophoblastic cells.

Mesothelial Cells.—In three samples of pulmonary vein blood we have found abundant cells of the type shown in Fig. 18. Comparison with the cells of pericardial fluid obtained during thoracic operations strongly suggests that these are free mesothelial cells accidentally included in the blood samples as a result of the needle traversing the pericardium. They are free cells of pericardial fluid rather than cells detached by the needle.

Features of Malignant Cells

The identification of malignant cells in any situation depends on two main characteristics. Firstly, the cells concerned must differ substantially from the other cell types found in that situation. In the case of white cell concentrates this requires a thorough knowledge of the cells of the blood including the more unusual ones described above. Secondly, the cells in question must correspond to the known appearance of malignant cells. As the cells of malignant tumours are extremely variable, so that it can almost be said that each tumour has a cell type unique to itself, the second criterion depends on a large experience of free malignant cells examined by the same technique.

At the best, the identification of a malignant cell on morphological grounds is only an opinion, and cannot be verified. The observer has to be constantly aware of his own fallibility. In the whole of our series, excluding leukaemia, myeloma, and

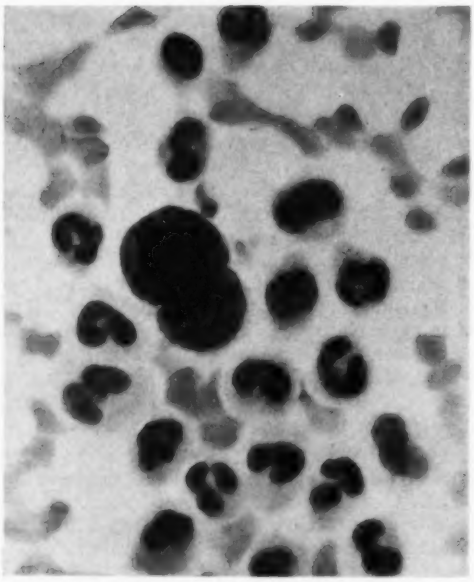

FIG. 5.—Megakaryocyte nucleus in a white cell concentrate fixed and stained by the Papanicolaou technique. Case of myelosclerosis (×1,300).

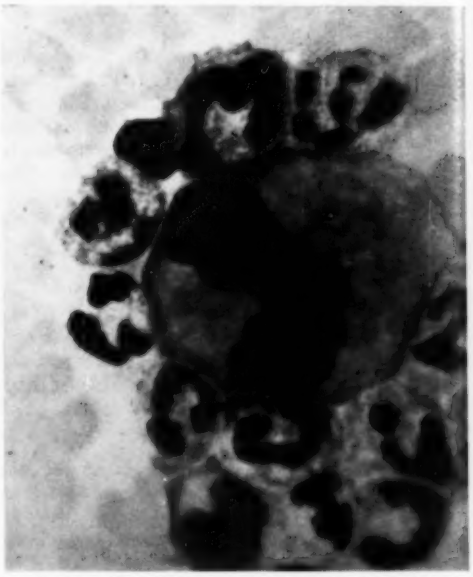

FIG. 6.—Complete megakaryocyte in the blood of a patient with multiple myeloma. Megakaryocytes with complete cytoplasm are much less common than the loose nuclei shown in Figs. 4 and 5 (×1,300).

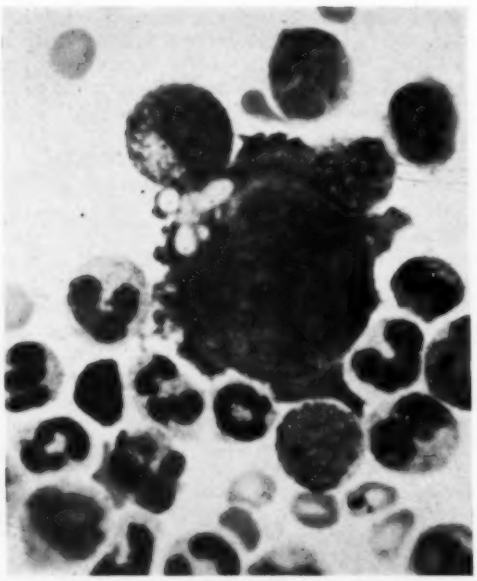

FIG. 7.—Megakaryoblast in the blood of a man of 51 with myelosclerosis. The cytoplasm was stained deep blue and showed no sign of the pink granulation which is present in mature megakaryocytes (×1,300).

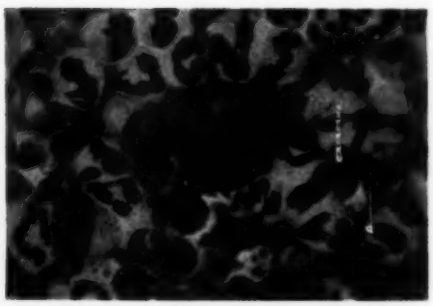

FIG. 8.—Another megakaryoblast from the same case as Fig. 7. Note the narrow rim of cytoplasm, which is more deeply basophilic than that of most tumour cells (×485).

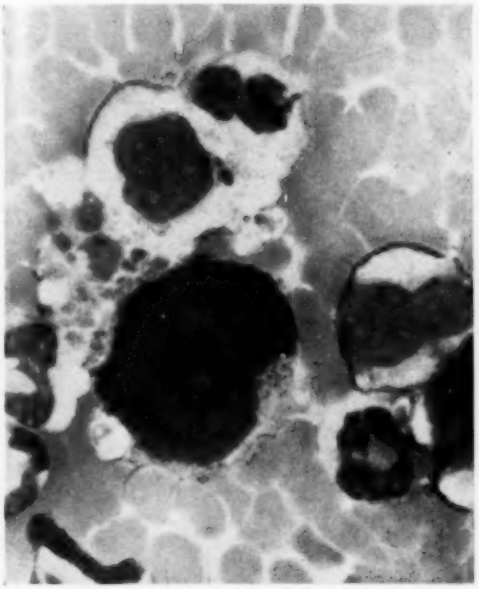

FIG. 9.—Immature megakaryocyte in the blood of a man aged 58 with myelosclerosis. This shows a group of adherent platelets (×1,300).

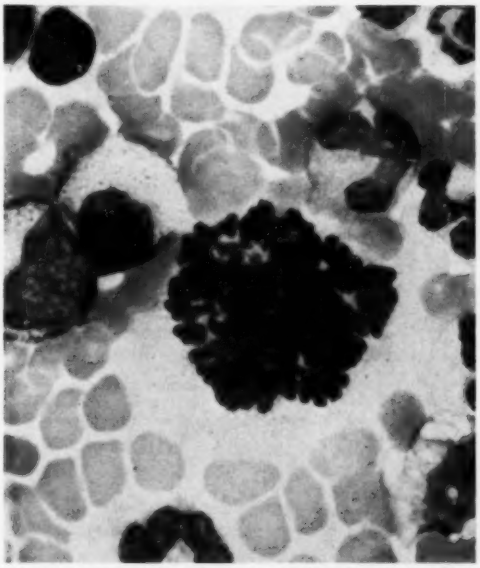

FIG. 10.—Same case as Fig. 9; a megakaryocyte is in mitosis (? multipolar) in the circulating blood (×1,300).

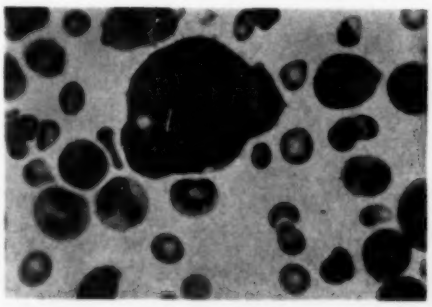

FIG. 11.—Immature megakaryocyte in the blood of a woman with myelosclerosis. Note the high nucleo-cytoplasmic ratio and dark blue cytoplasm (×485).

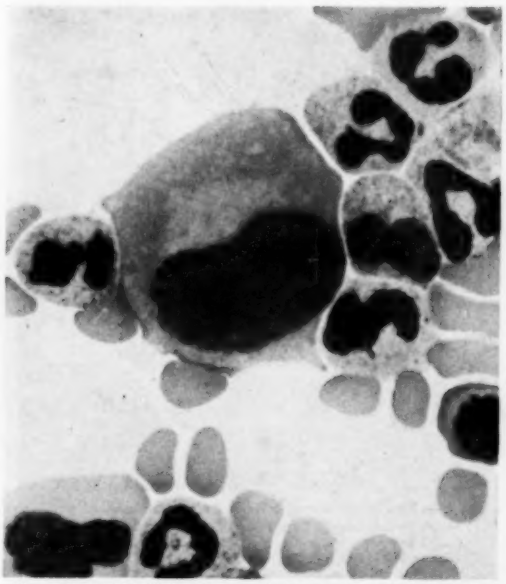

FIG. 12.—Unidentified cell from the same case as Figs. 7 and 8. This might belong to the megakaryocyte series. The patient did not have malignant disease (×1,300).

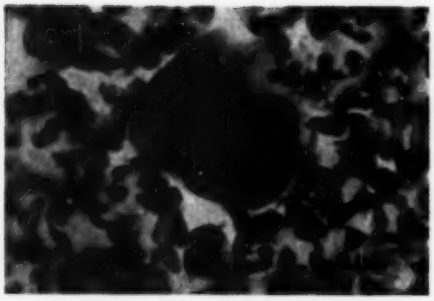

FIG. 13.—This cell is most probably an osteoclast. It comes from the pulmonary vein blood of a patient with carcinoma of the lung. Note the multiple uniform oval nuclei (×485).

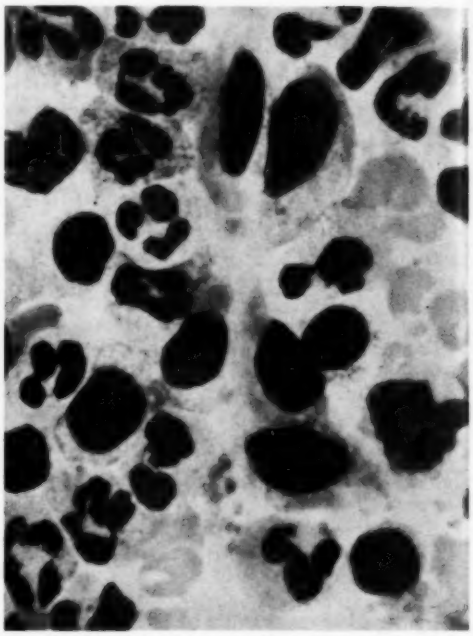

FIG. 14.—Endothelial cells, together with neutrophils and lymphocytes, in venous blood from a case of melanoma. The nuclei are elongated and the cytoplasm stains pale blue (×1,300).

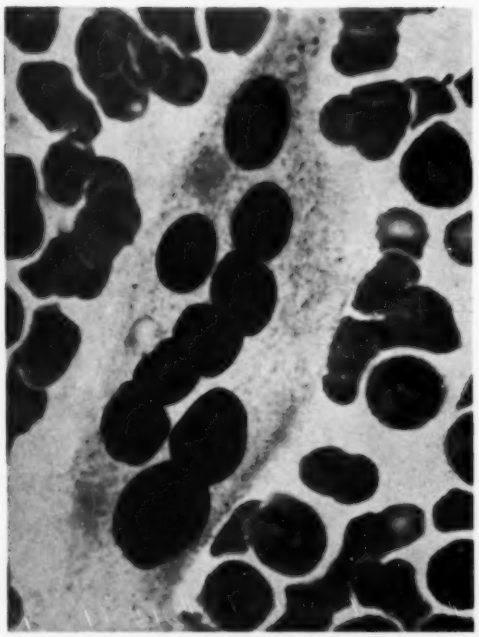

FIG. 15.—Multinucleate endothelial cell in venous blood. All the nuclei in this cell are pyknotic. Giant cells of this type can be found in smears made direct from vascular endothelium. Same case as Fig. 14 (×1,300).

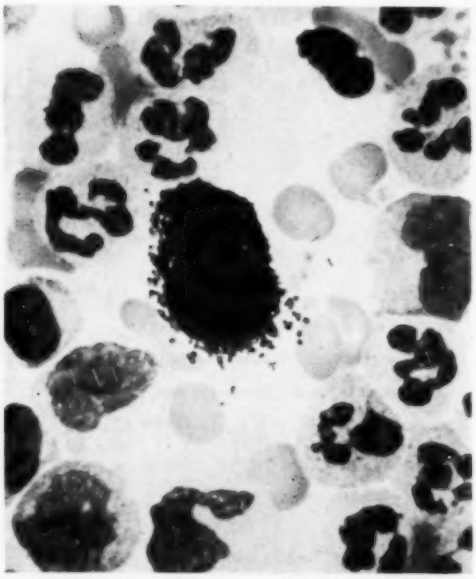

FIG. 16.—Tissue mast cell, from venous blood of a case of carcinoma of the lung (×1,300).

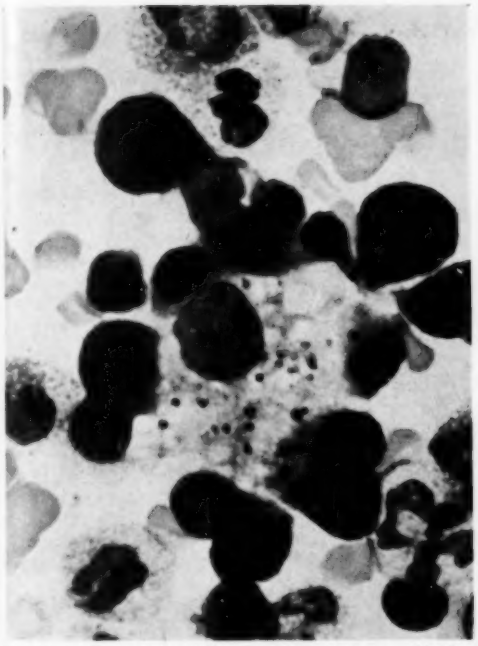

FIG. 17.—Erythropoietic embolus from antecubital venous blood of a patient with multiple myeloma (same case as Fig. 6). There is a central macrophage or reticulum cell containing pigment, surrounded by a ring of normoblasts (×1,300).

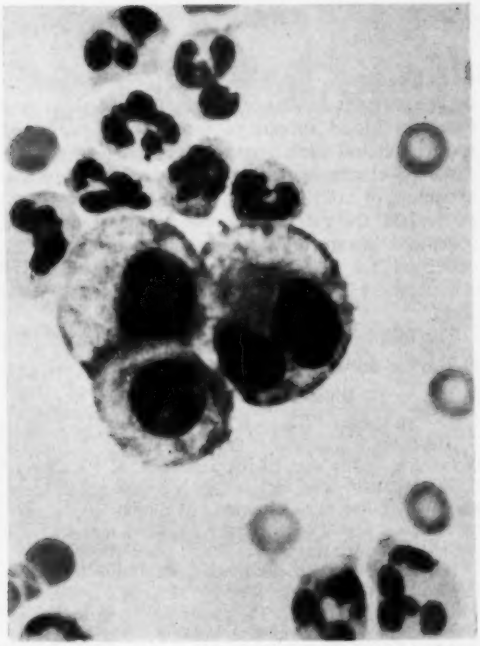

FIG. 18.—Mesothelial cells. The blood sample was obtained from the pulmonary vein of a patient with carcinoma of the lung. These cells were probably accidentally introduced, as the needle traversed the pericardium (×1,300).

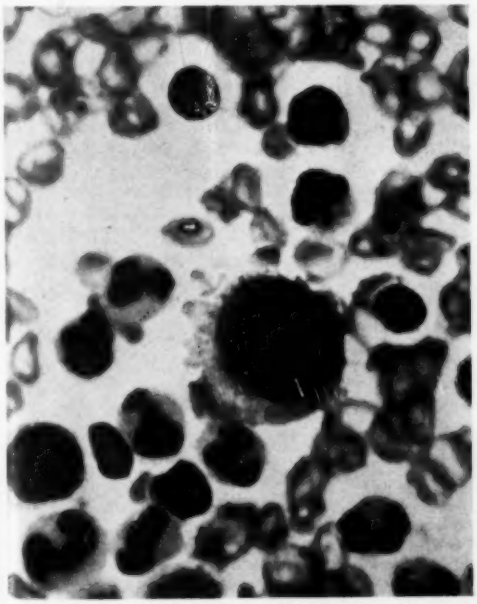

FIG. 19.—Tumour cell from a patient with malignant melanoma and multiple metastases (Case 1). This cell contained a small amount of fine melanin pigment. Similar cells were present in peritoneal fluid (×1,300).

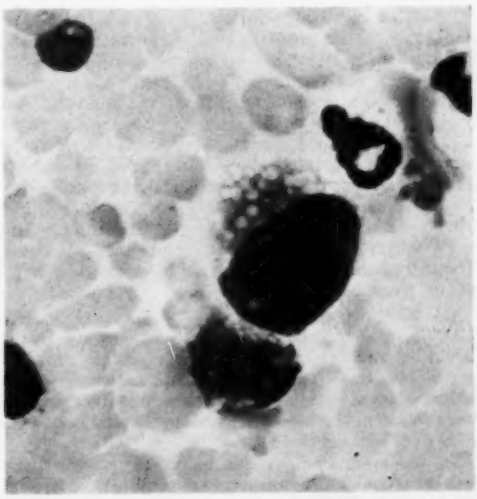

FIG. 20.—Tumour cell from a patient with malignant synovioma and widespread metastases (Case 2). Similar cells were found in bone marrow, pleural fluid, and cerebrospinal fluid (×1,300).

reticulosarcoma, we have only found acceptable malignant cells in seven cases. Two of these had extensive blood spread of sarcomas, a malignant synovioma and a malignant melanoma. Of the five cases of carcinoma, three had carcinoma of the bronchus, one of the breast, and one of the stomach. In the last, the specimen was obtained from a local vein, and in one of the cases of carcinoma of the bronchus the blood came from the pulmonary vein; otherwise all were venous samples from the arm.

The following are abbreviated case histories with descriptions of the cells.

Illustrative Case Reports

(1) **Malignant Melanoma.**—D. A. (Reg. No. 25563), a 33-year-old woman, with a pigmented malignant melanoma of the skin of the abdomen, at the time of finding tumour cells in the blood had hepatomegaly, anaemia, numerous scattered slate-blue spots, and melanuria. She died five months later, and multiple blood-borne metastases were found at necropsy.

The antecubital venous blood concentrate showed a few isolated tumour cells (Fig. 19) 22 to 28 μ in diameter with nuclei 15 to 20 μ. The nuclei were central, round, with fine chromatin and blue nucleoli. The cytoplasm was pale blue and had irregular margins. Some cells contained small granules of pigment. There were also many macrophages with coarse melanin granules. (This case is described elsewhere in more detail by Goodall *et al.*, 1960.)

(2) **Malignant Synovioma.**—P. A. (Reg. No. 9629), a 16-year-old girl, had a malignant synovioma which had been excised from the ulnar border of the hand 18 months previously. At the time of finding tumour cells in the blood she had lesions in the cervical and lumbar spine, the pelvis, and lymph nodes of the neck. There was a leuco-erythroblastic anaemia (haemoglobin 50%, 7.4 g. per 100 ml.). Tumour cells were demonstrated in the cerebrospinal fluid, the marrow, and pleural fluid. She died 10 weeks later and necropsy confirmation was obtained. Tumour cells were fairly numerous in the antecubital venous blood concentrate and the cell illustrated (Fig. 20) was typical, measuring 26×21 μ, and the nucleus 21×15 μ. The nucleus was usually eccentric with slight irregularities and round clefts which stained pink, and had fine chromatin. The cytoplasm was of an opaque blue with a number of small vacuoles.

(3) **Carcinoma of the Bronchus.**—C. A. (Reg. No. 270081), a 66-year-old man, had had cough for nine weeks before thoracotomy, when an inoperable bronchial carcinoma was found. A mediastinal lymph-node biopsy showed anaplastic carcinoma, and tumour cells were found in the blood from an antecubital vein. He died three months later.

In the antecubital venous blood concentrate there was one clump of three tumour cells (Fig. 21), with diameters from 13 to 19 μ, showing a high nucleo-cytoplasmic ratio, irregularity, and mutual moulding

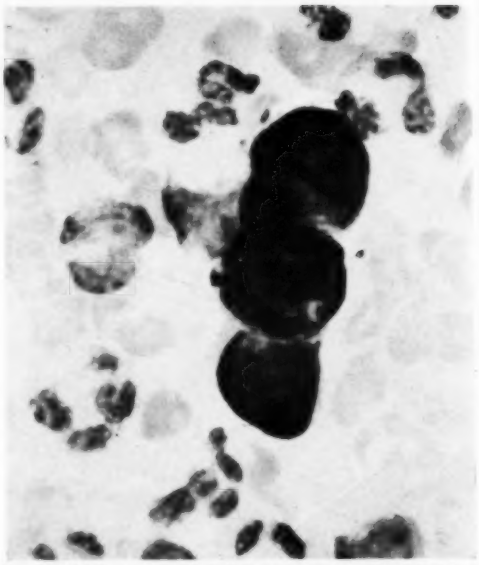

FIG. 21.—Malignant "oat-cells" from pulmonary vein blood in a case of carcinoma of the lung (Case 3) (×1,300).

of the nuclei as is commonly seen in the "oat cells" illustrated by Spriggs (1957).

(4) **Carcinoma of the Bronchus.**—H. J. (Reg. No. 201020), a man aged 64 years, had had a stroke three months previously and pain in the chest one month before the finding of tumour cells in the blood. At that time he had a leuco-erythroblastic anaemia, a pleural effusion, hepatomegaly, jaundice, and a massive crop of small skin metastases which had developed in the previous 24 hours. He died three days later. Necropsy showed carcinoma of the right lower lobe bronchus, enlarged hilar nodes, and vertebrae riddled with deposits.

The antecubital venous blood concentrate showed a variety of abnormal cells. As well as the immature

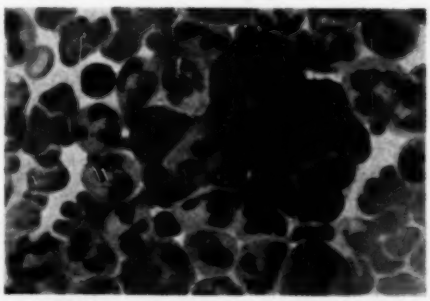

FIG. 22.—Malignant "oat-cells" from antecubital venous blood of a patient with carcinoma of the lung and very numerous metastases (Case 4). Note the mutual compression of nuclei and scanty pale-staining cytoplasm (×485).

haemopoietic cells, there were megakaryocytes or their bare nuclei, several macrophages, some containing pigment and a few containing two or three normoblasts at different stages of development, as well as unrecognizable pigmented material. The ingested normoblasts were well preserved. The macrophages varied from monocytes to larger cells with oval nuclei and profuse vacuolated cytoplasm sometimes finely dusted with eosinophilic matter. The variations and gradations from one type of cell to another were remarkable.

There were also numerous tumour cells, frequently in clumps of two or three and some up to 14 in number (Fig. 22). Single cells of similar appearance were present. The tumour cells were mostly 15 to 17 μ in diameter, occasionally larger. The nuclei showed fine chromatin, a high nucleo-cytoplasmic ratio, and the mutual moulding of oat cells.

(5) **Carcinoma of the Bronchus.**—J. R. (Reg. No. 259099) was a 64-year-old man with carcinoma of the bronchus. The diagnosis was based on haemoptysis, the clinical findings, a lung shadow on radiographs, and the bronchoscopic findings. At the time of finding tumour cells in the blood he had headaches, neurological lesions, and superior vena caval obstruction. He died two weeks later. No biopsy or necropsy was obtained.

The antecubital venous blood concentrate showed five clumps of from four to eight tumour cells (Fig. 23).

FIG. 23.—Anaplastic malignant cells from antecubital venous blood of a case of carcinoma of the lung (Case 5). These cells are larger than "oat-cells" and have a more profuse cytoplasm which stains pale blue-grey (×1,300).

These measured about 30×25 μ but the cell margins were ill-defined. The irregular, angular nuclei measured 17 to 28 μ in diameter. There were small separate nuclear fragments, the chromatin was finely punctate, and nucleoli were faintly seen. A few small vacuoles were present in the nuclei as well as the cytoplasm, which stained pale blue of varying density.

(6) **Carcinoma of the Breast.**—E. G. (Reg. No. 10270), a 36-year-old woman with scirrhous carcinoma of the breast, had had a radical mastectomy two and a quarter years previously. At the time of finding tumour cells in the blood she had diffuse bony metastases and a leuco-erythroblastic anaemia. Haemoglobin was 56% (8.3 g. per 100 ml.). Sternal marrow aspiration showed deposits of cells similar to those found in the peripheral blood. She died five months later.

The antecubital venous blood concentrate showed two rounded clumps and some single tumour cells. The larger clump had about 80 cells of 20 μ diameter (Fig. 24). The oval or round nuclei varied in size from 10 to 15 μ, some having irregularities and several having blue nucleoli 3 μ in diameter. The cytoplasm, of a finely granular blue, often showed no distinct cell margin.

(7) **Carcinoma of the Stomach.**—F. H. (Reg. No. 270542) a 59-year-old man with carcinoma of the stomach, had had symptoms for nine weeks before operation when tumour cells were found in the blood. Similar cells had been demonstrated in the gastric aspiration. Biopsy showed a poorly differentiated adenocarcinoma.

FIG. 24.—A cluster of tumour cells from antecubital venous blood of a case of carcinoma of the breast with widespread metastases. The cells occur in rounded clumps, unlike the ragged sheets formed by endothelial cells. Identical clumps were present in the bone marrow (Case 6) (×260).

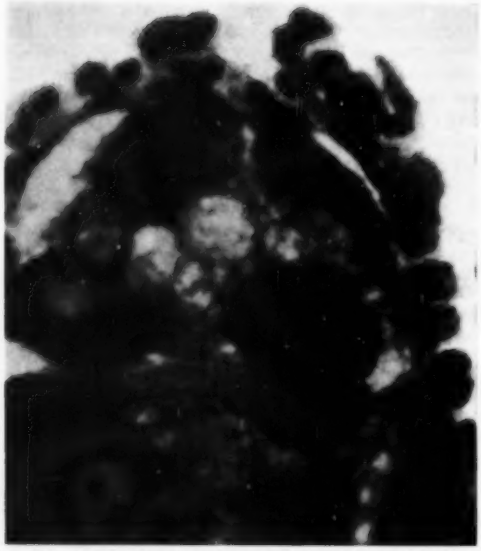

FIG. 25.—A collection of tumour cells from regional venous blood draining from a carcinoma of the stomach. A portion of the clump is shown, with a rounded edge. The cells composing the clump are distorted by vacuoles. Similar structures were found in gastric washings from this patient (Case 7) (×1,300).

Death occurred four weeks later following pulmonary embolism from calf veins. Necropsy further demonstrated a large carcinomatous ulcer encircling the pylorus and tumour deposits in the gastric lymph nodes, the peritoneum, and the liver.

An omental venous blood sample showed several clumps of up to 12 tumour cells (Fig. 25). These cells were 30 to 40 μ in diameter with irregularly shaped nuclei between 15 and 40 μ across. Vacuolation with distortion of the cells was frequent.

Conclusions

Our findings confirm the fact that malignant cells can be recovered from circulating blood in certain cases of sarcoma and carcinoma. In this series there are seven cases in which such cells have been identified. None of the patients survived more than a few months after the first appearance of the cells, and we have failed to demonstrate any malignant cells in the blood of patients with operable carcinoma, even using samples from the venous drainage of the tumour.

Several other types of cell large enough to cause confusion with metastatic tumour cells are found occasionally or in small numbers in the blood. In normal healthy persons there are only a few mature megakaryocyte nuclei, and normal samples are therefore inadequate as control material from which to study the lesser known non-malignant cells. Diseases accompanied by extramedullary blood formation, such as myelosclerosis, are an excellent source of puzzling cells, and we have illustrated megakaryoblasts from the peripheral blood of patients with this condition, which, if they had been seen in a case of advanced carcinoma, might well have been called malignant. Carcinomatosis, when it involves the skeleton, often causes a similar blood picture, and this can easily be a source of error.

Again, plasma cells and their precursors are well known to be increased in number in malignant disease, and it is necessary to be familiar with their variations.

Most of the unusual cells illustrated in the plates came, in fact, from cases of carcinoma, and it cannot be emphasized too strongly that histological proof of malignant disease on follow-up does not constitute sufficient proof that a "positive" cytological finding was correct. There is still room for further research into the non-malignant cells occurring in the circulation before statistical data on the frequency of circulating tumour cells can be considered accurate.

We are grateful to our colleagues of the United Oxford Hospitals who have kindly obtained samples of blood for this study; also to Dr. A. H. T. Robb-Smith for reading the manuscript and to Mr. M. M. Boddington, M.A., B.Sc., for help during the investigation and with the photographs. One of the authors (A. I. S.) gratefully acknowledges a grant from the British Empire Cancer Campaign.

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THE DIAGNOSTIC VALUE OF PLEURAL BIOPSY IN BRONCHOPULMONARY CARCINOMA

BY

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The diagnosis of bronchopulmonary carcinoma was either corroborated or arrived at in 13 out of a total of 26 cases by pleural biopsy. Taken as a whole, pleural biopsy is inferior to repeated examination of the pleural exudate for neoplastic cells. In individual observations, however, the former can be found positive at a time when neither the pleural fluid nor any other material provide a diagnostic clue.

In an instance of primary pleural growth (mesothelioma) its nature was recognizable in the pleural biopsy material.

Pleural biopsy can correctly lead to the diagnostic exclusion of growth in favour of tuberculosis.

Pleural biopsy can be suggestive of the rheumatic aetiology of changes.

A pleural biopsy positive for carcinoma can be obtained on both sides.

All stages, from early submesothelial deposition of individual neoplastic cells to the dense infiltration of the subserous connective and fat tissue, were observed.

The diagnostic usefulness of pleural needle biopsies has been pointed out on several occasions (Hill, Hensler, and Breckler, 1958; Mestitz, Purves, and Pollard, 1958; Leggat, 1959; Karlish, 1959a, b). The results were particularly impressive in the large series (228 biopsies) presented by Mestitz *et al.* (1958).

However, no unanimity has been reached as to the real usefulness of the method and this seems to be the reason why it has not yet been adopted generally and routinely. Informative individual case reports are therefore still called for, and those given in the present paper from a comparatively small series largely confirm the favourable view arrived at before. However, while helpful in certain instances, the method does not appear to be indispensable and not infrequently fails at a time when other diagnostic methods, such as the examination of pleural fluid and bronchial biopsy, have led to the desired result.

The Present Series

The series comprises a total of 26 cases in which pleural biopsy was performed uniformly with the Adams (or Harefield) needle on one or several occasions for the diagnosis of bronchopulmonary carcinoma. It was positive in 13 cases and negative in 13.

In all 13 positive cases the diagnosis was confirmed: in six by bronchial biopsy, in four by suggestive bronchoscopic findings, and in six by examination of the pleural fluid and/or sputum for neoplastic cells. Of the 13 negative pleural biopsies, seven were in cases of carcinoma.

It should be expected that a positive pleural biopsy implies the presence of neoplastic cells in the pleural fluid, and indeed the longer and more often this is searched the higher will be the yield of neoplastic cells. However, the pleural fluid can be found devoid of neoplastic cells at a time when the pleural biopsy does reveal carcinoma. This was found in two cases of the present series.

Against this there were three cases in which the reverse was seen, namely, neoplastic cells in the pleural fluid with a negative pleural biopsy. The latter event should be more common in view of the proximity of many lung tumours to the visceral pleural lymphatics. In our small series pleural biopsy was thus indicative of bronchopulmonary carcinoma in about half the cases and failed to do so in roughly one-fourth. It therefore compares unfavourably with examination of the pleural exudate which failed only in one-fifth of the cases (in 12 out of 67 cases) examined at Clare Hall Hospital in 1957–59.

Case Reports

Of the individual cases the following are noteworthy:

(1) A man, aged 56 years, with one month's history of pain in the chest, cough, and dyspnoea, presented with a right pleural effusion that was aspirated several times. Bronchoscopy showed stenosis of the right upper lobe bronchus, but the bronchial biopsy was negative. A subsequent pleural biopsy was positive.

In this instance the positive pleural biopsy formed the only pathological evidence of carcinoma. On its strength no further investigations were made, as the radiological extent of the growth and the general condition of the patient rendered it inoperable.

(2) In a man, aged 62 years, with four months' history of cough, sputum, and dyspnoea, a left pleural effusion was found on *x*-ray examination. This was aspirated several times. On bronchoscopy narrowing of the left stem bronchus was found. Both the bronchial biopsy and pleural fluid were negative. Pleural biopsy was, however, positive.

Squamous-celled carcinoma was diagnosed from the pleural biopsy on two occasions at a time when no evidence was forthcoming from the bronchial biopsy and the pleural fluid. Bronchoscopically, however, a suggestive stenosis was seen.

(3) This patient, a man aged 67, had a two years' history of hoarseness of the voice. He then developed gastric symptoms for which he was investigated. Radiographs showed a large mass in the right upper zone. Bronchoscopy showed a narrowing of the lower trachea and stenosis of the right main bronchus. Bronchial biopsy did not reveal any neoplastic changes. The pleural biopsy, however, contained units of squamous-celled carcinoma.

This case was almost a replica of the previous one. Carcinoma was found in the pleural biopsy, with a negative bronchial biopsy in spite of bronchoscopic findings suggestive of bronchial stenosis.

(4) In a man, aged 57 years, with six months' history of cough, dyspnoea, and haemoptysis, the chest radiograph showed multiple bilateral opacities. There was a small right pleural effusion. Oat-celled carcinoma was found in several pleural biopsies and in an enlarged right supraclavicular lymph node, but no neoplastic cells were seen in the sputum.

In this instance a positive pleural biopsy antedated any other positive findings by about one month.

(5) In a man, aged 59 years, with progressive dyspnoea for two years a left pleural effusion was diagnosed. Though numerous aspirations were performed, only later were neoplastic cells found in the fluid. No changes could be seen on bronchoscopy. The needle biopsy was, however, positive (Fig. 1). Later, following an aspiration, he developed a pneumothorax. Radiographs taken at that time showed deposits on the thoracic wall.

In this case a growth that was most probably pleural in origin was discovered by pleural biopsy. The naked-eye appearance was of multiple, patchy, intrapleural deposits of a white growth (Fig. 2). Involvement of lung tissue was not demonstrable, either by naked eye or by histological examination, nor could any intrabronchial deposit be detected in a narrow series of sections through the specimen.

Histologically the growth consisted of tubular pseudo-adenomatous units showing central clefts lined by low cuboidal cells with scanty mitoses (Fig. 3). The growth conformed in type with what has been described as pleural mesothelioma or primary pleural tumour (Eisenstadt, 1956; Smart and Hinson, 1957; Forse and Haug, 1958). Particular reference should be made to the cases collected by McCaughey (1958) and his Figs. 10 and 11. In the present instance neither papillary structures nor neoplastic pleomorphic spindle-celled stroma, as often seen in these tumours, could be found.

Bronchoscopy and bronchial biopsy had been negative, but neoplastic cells were observed in the pleural fluid, though only after the diagnosis had been made from the pleural biopsy.

(6) In a man, aged 67 years, a pleural effusion containing neoplastic cells (Fig. 4) developed six months after a negative chest radiograph. Bronchoscopy showed narrowing of the left stem bronchus. A bronchial biopsy, however, was negative, whereas a pleural biopsy showed carcinoma (Fig. 5).

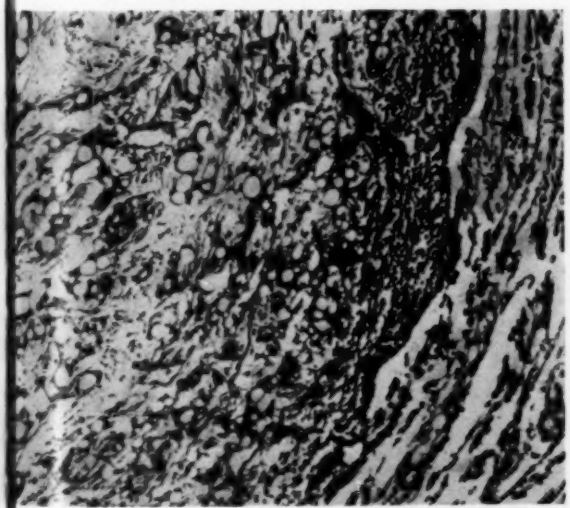
A case of oat-celled carcinoma of the bronchus proved by pleural biopsy on two occasions, on which neither the bronchial biopsy nor the sputum provided any evidence. On the other hand, such evidence was forthcoming from the pleural fluid.

(7) In a man, aged 68 years, with a history of chronic bronchitis and recent haemoptysis, the sputum was found to contain neoplastic cells (Fig. 6). After pleural effusion had developed, squamous-celled carcinoma was proved by pleural biopsy (Fig. 7), and a neoplastic mass blocking the left stem bronchus was seen bronchoscopically. Bronchial biopsy confirmed the pleural biopsy findings (Fig. 8).

This observation is reported in detail because of the scantiness of carcinomatous units in the pleural biopsy, calling for a careful and prolonged search, including cytological examination under oil immersion. In the present instance only one

FIG. 1.—Case 5: Primary pleural growth (mesothelioma) in a man aged 59. Pleural biopsy × 100 showed multiple clefts lined by uniformly low cuboidal "epithelia."

FIG. 2.—Case 5: Specimen showing multiple patchy deposits in the visceral pleura.



3.—Case 5: Histological aspects of growth × 100. The same pattern of clefts lined by "epithelium" as seen in Fig. 1, limited to visceral pleura, encroaching on but not infiltrating lung.

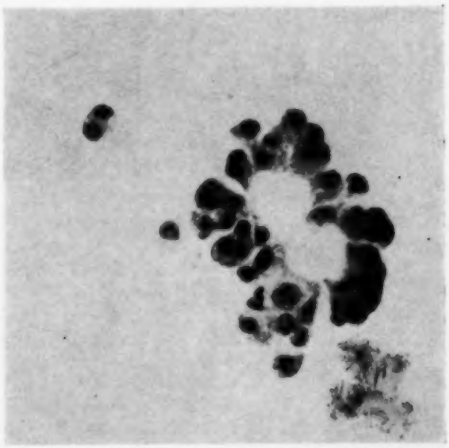


FIG. 4.—Case 6: Positive pleural, negative bronchial biopsy in a man aged 67. Neoplastic unit from pleural exudate × 400.

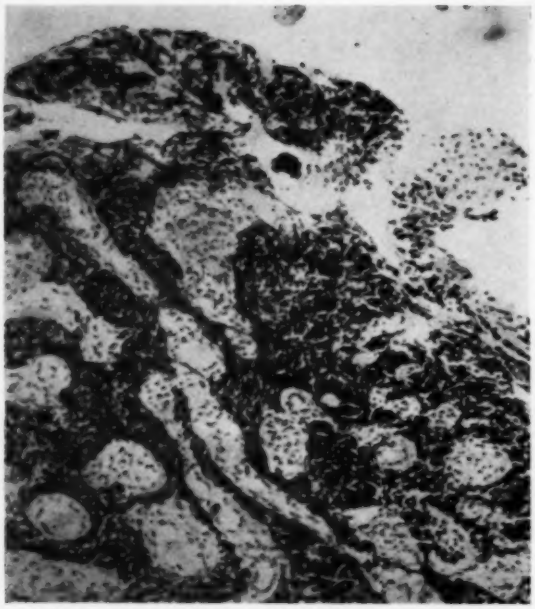


FIG. 5.—Case 6: Pleural biopsy × 100. Dense infiltration of subserous fat tissue with oat-celled carcinoma.

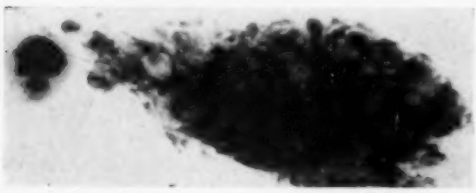


FIG. 6.—Case 7 illustrating scantiness of carcinoma cell units in pleural biopsy in a man aged 68 years. Neoplastic cells in sputum × 400.

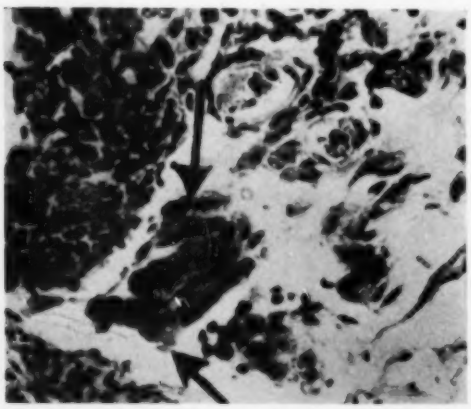


FIG. 7.—Case 7: Pleural biopsy × 400 showing scanty units of carcinoma (arrows).

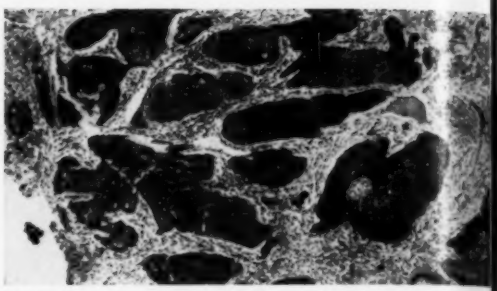


FIG. 8.—Case 7: Bronchial biopsy × 40 showing squamous-cell carcinoma.

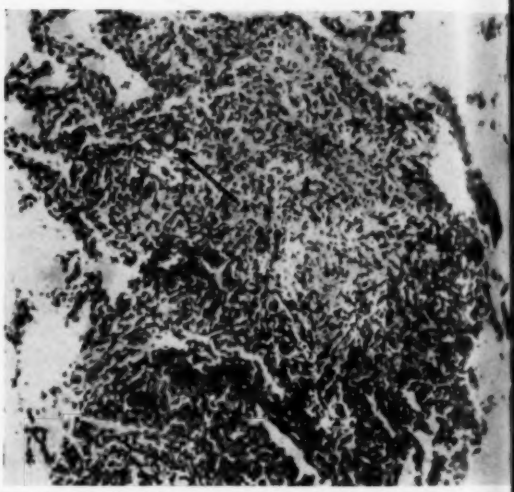


FIG. 9.—Case 8: Pleural biopsy with tuberculous changes in a woman aged 35. Pleural biopsy × 100, "clearance" area. Peripherally a giant cell (arrowed) of the Langhans type.

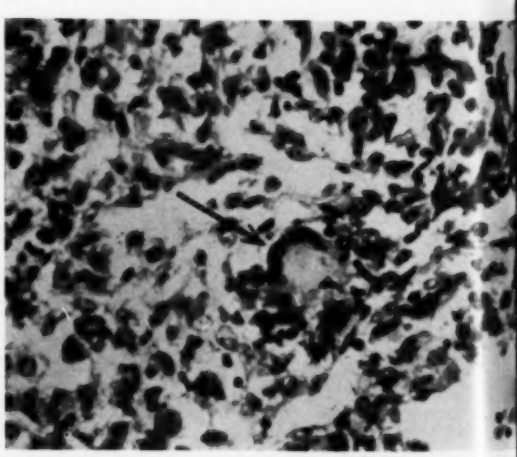


FIG. 10.—Case 8: The same as Fig. 9. Close-up of giant cell × 400

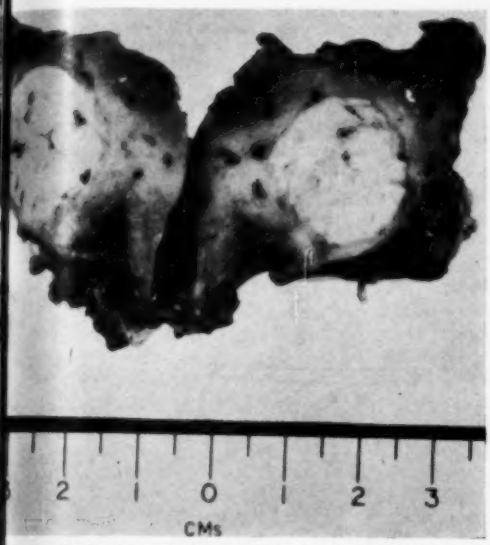


FIG. 11.—Case 8: Specimen of tuberculoma in the right lower lobe with marginal epituberculosis.

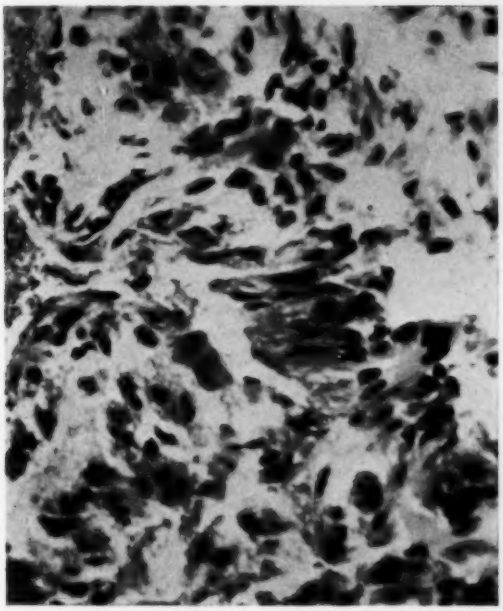


FIG. 13.—Case 9: Second pleural biopsy taken eight days later showing "palisading" of cells with tendency to confluence (syncytia) × 400.

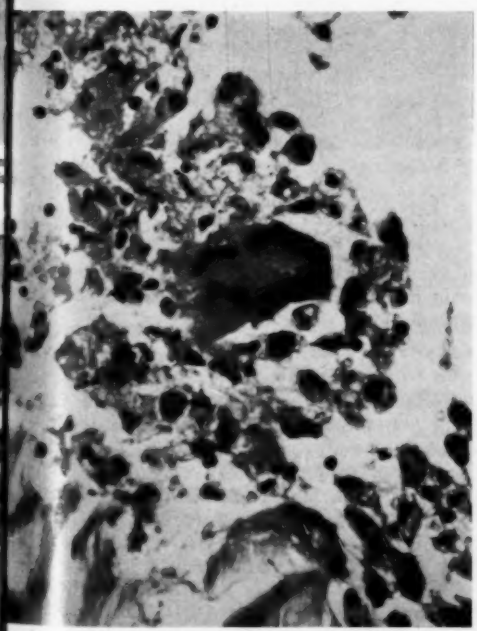


FIG. 12.—Case 9: Pleural biopsy suggesting rheumatic aetiology in a man aged 53 years. Macrophages and a giant cell of the Langhans type × 400.

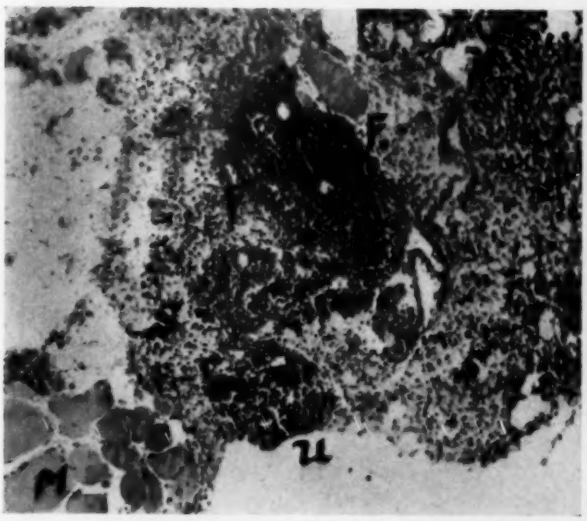


FIG. 14.—Case 4: Pleural biopsy × 100 in a man aged 57 years. Endolymphatic unit (U) and scattered neoplastic cells (S), M muscle, F fibrin clot.

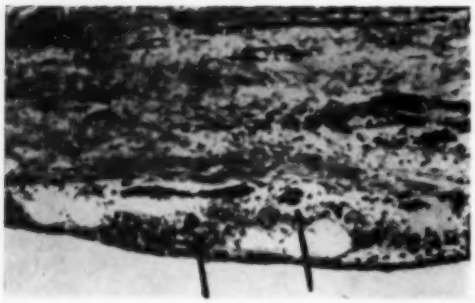


FIG. 15

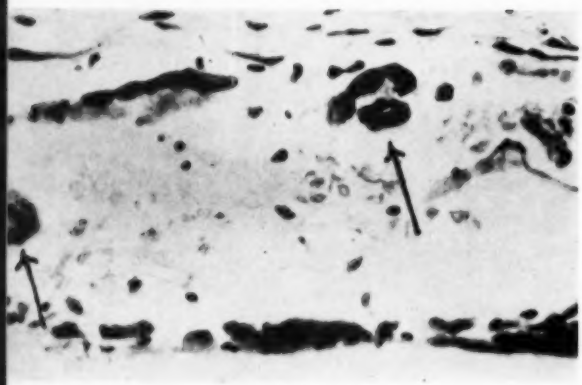


FIG. 16

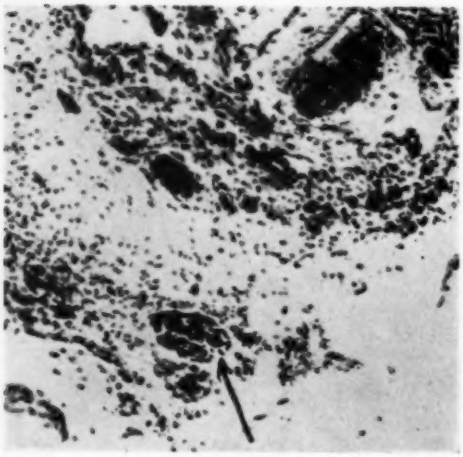


FIG. 17

FIG. 15.—Case 11: Pleural biopsy showing the earliest stage of micro-deposit in the parietal pleura, one individual and a small cluster of neoplastic cells in a submesothelial lymph space (arrows) × 100. The mesothelial strip forms the extreme outer margin of the picture.

FIG. 16.—Case 11, the same as in Fig. 15 × 400.

FIG. 17.—Case 11: Stage subsequent to that shown in Figs. 15 and 16. Two solid units of carcinoma are shown in a deep lymph space (arrow) × 100.

FIG. 18.—Case 11: The same as Fig. 17 × 400.

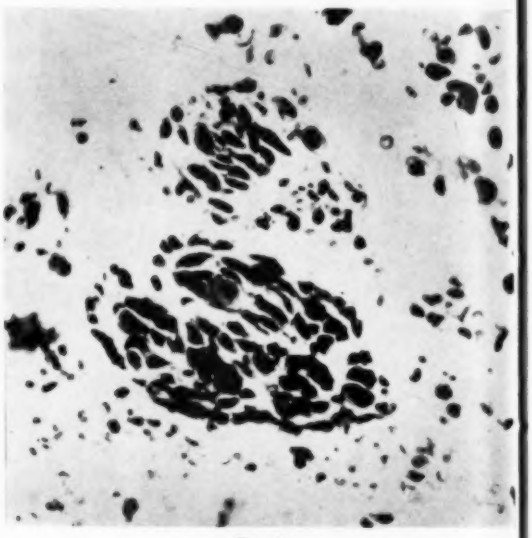


FIG. 18

such unit was found in many sections examined. It was situated in a lymph space. Before the positive pleural biopsy, neoplastic cells had been seen in the sputum, but only later was a positive bronchial biopsy obtained.

Discussion

From the present series, however small, it is evident that pleural biopsy can be useful in (*a*) corroborating the diagnosis of bronchopulmonary carcinoma suggested or arrived at by other means; (*b*) rendering bronchial biopsy unnecessary; (*c*) furnishing a positive result in a case with a negative bronchial biopsy. As a rule persistent search for neoplastic cells in the pleural fluid will eventually lead to a positive result without pleural biopsy. The latter may be attempted, however, in the absence of exudate and lead to a positive result. This was seen in the only case of the present series in which the tuberculous nature

of a mass in the lung was correctly suggested by pleural biopsy. The patient was a woman, aged 35 (Case 8), with a shadow suspicious of carcinoma in the right lower lobe, in spite of some history of tuberculosis treated with drugs. A pleural biopsy showed chronic inflammatory changes around an area of "clearance" and fibrosis with a solitary large giant cell of the Langhans type at the periphery (Figs. 9 and 10). The segment of the right lower lobe containing the suspicious change was then removed. It contained a tuberculoma (Fig. 11) with a broad peripheral zone of chronic collapse containing many non-caseating tuberculoid nodules that were rich in giant cells of the Langhans as well as the foreign body type. Both the naked-eye and histological changes closely resemble those observed in epituberculosis (Pagel, Simmonds, and Macdonald, 1953) and in this instance are due to chemotherapy.

The presence in a pleural biopsy of giant cells of the Langhans type in itself is no proof of tuberculosis. This is illustrated by a case in which pleural biopsy suggested a rheumatic aetiology. A man aged 53 years (Case 9), with recurrent multiple rheumatoid arthritis of 16 years' standing, developed, during a period of exacerbation, a unilateral pleural effusion. A pleural biopsy showed large mononuclear cells with a tendency to form giant cells of the Langhans type (Fig. 12). In a second pleural biopsy taken eight days later no giant cells of the Langhans type were seen, but there was distinct palisading of mononuclear cells with a tendency to form syncytia (Fig. 13). The changes were reminiscent of those seen in the cellular parts of rheumatic infiltrates. A suspicion of tuberculous aetiology (as suggested by the first pleural biopsy) could not be confirmed by clinical, radiological, or bacteriological evidence.

A pleural biopsy positive for carcinoma can be obtained on both sides. This was seen in a man aged 52 years (Case 10) with a shadow suspicious of growth in the right lower zone of the chest. The pleural exudate from both sides contained many neoplastic-looking cells. Pleural biopsies taken from both sides likewise showed units of carcinoma.

In the present series, in all positive cases a real infiltration of the pleural membrane by neoplastic cells or units was demonstrated compared with a merely superficial adhesion of neoplastic units deposited from the pleural fluid. The pictures observed fall into three categories: (*a*) Deep-seated and extensive infiltration of the subserous fat tissue (Fig. 5); (*b*) patchy units lying in isolated lymph spaces, sometimes scanty and difficult to trace (Fig. 7); (*c*) superficial, but submesothelial infiltration, of isolated neoplastic cells or small groups mixed with blood or fibrin (Figs. 14, 15, and 16). The latter pictures illustrate the very early stages in the involvement of the pleural membrane in a marginal submesothelial lymph space. This is followed by larger units collecting in lymph spaces (Figs. 17 and 18). Both these stages were seen in two pleural biopsies taken simultaneously in a man aged 46 with squamous-celled carcinoma of the right main bronchus with pleural effusion (Case 11): the second stage was close to the growth itself where it was adherent to the parietal pleura, whereas the earlier changes of superficial submesothelial infiltration were found in a pleural strip remote from the former site.

A point of more general interest that emerges from the examination of pleural biopsies in bronchopulmonary carcinoma is the comparatively frequent presence of deposits in the parietal pleura. Perhaps this is less surprising in view of the pleural effusion that is present in most cases in which pleural biopsy is attempted, indicating an early involvement of the visceral pleura. Nevertheless the common occurrence of micro-deposits in the parietal membrane appears to be a subject worthy of systematic clinicopathological studies, with particular reference to the prognostic significance of such deposits.

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THREE CASES OF ACUTE MESENTERIC LYMPHADENITIS DUE TO *PASTEURELLA PSEUDOTUBERCULOSIS*

BY

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Seventeen cases of mesenteric adenitis were investigated between May and October, 1959, for the presence of virus as well as for evidence of *Pasteurella pseudotuberculosis* infection.

Specimens examined included mesenteric glands, appendix, throat swab, faeces, and serum, although glands were received from only 12 patients. Virus was not isolated from any of the specimens, but evidence of infection with *Pasteurella pseudotuberculosis* was obtained in three patients.

The three cases of acute mesenteric lymphadenitis due to *Pasteurella pseudotuberculosis* are reported.

Pasteurella pseudotuberculosis Type IA was isolated from the mesenteric glands of two of the cases, and all three cases showed serological evidence of infection with the organism. The mesenteric glands of two of the cases showed histological changes characteristic of pseudotuberculosis.

Acute mesenteric lymphadenitis, mimicking appendicitis, is not uncommon in this country. Aird (1945) reports that in the Royal Hospital for Sick Children, Edinburgh, during the year 1944, there were admitted in his charge 37 patients suffering from non-specific mesenteric adenitis compared with 83 suffering from acute appendicitis and four suffering from abdominal tuberculosis. During the period May to October, 1959, of 93 children admitted for suspected appendicitis to the Leicester Royal Infirmary, 20 were found to be suffering from acute mesenteric adenitis.

Many causes have been suggested, including infection with organisms of the dysentery group (Felsen, 1935), infection with a hypothetical virus derived from the upper respiratory tract (Aird, 1945), and hyperinfestation of the intestine with roundworms (Kirthi Singha, 1959).

In 1953, Masshoff and Dölle observed certain pathological changes in the mesenteric glands of young children operated on for suspected appendicitis. At operation the appendix was found to be normal or only slightly inflamed while the ileocaecal glands were enlarged. The changes in the glands were characterized by the formation of one or more follicles consisting mainly of reticulum cells. Because of the tendency of the follicles to undergo necrosis the authors described the pathological process that they had observed as an "abscess-forming reticulocytic lymphadenitis," and suggested that it might be of viral origin, since the histological changes in the affected glands resembled those found in cat-scratch fever and lymphogranuloma inguinale. In the following year, however, Knapp (1954) and Knapp and Masshoff (1954) reported the isolation of *Past. pseudotuberculosis* from the enlarged mesenteric glands of two children operated on for appendicitis. In both cases the appendix was normal. The histological changes in the glands were identical with those described by Masshoff and Dölle. From then until 1957, 117 cases of *Past. pseudotuberculosis* were diagnosed by Knapp and his co-workers at the Hygiene-Institut in Tübingen (Knapp, 1958).

Early in 1959 Dr. G. S. Wilson drew our attention to the work of Knapp and his colleagues, and as we were already making an investigation into the possible viral origin of mesenteric adenitis, it was decided to include *Past. pseudotuberculosis* in the scope of our inquiry.

Arrangements were made to collect glands, appendix, faeces, throat swab, and serum from each patient. In the six months from May, 1959, we received the full complement of specimens from 12 patients, and from five others all the material with the exception of the glands. Bacteriological and histological examinations were made of the glands and appendices, and the

sera were examined for antibodies to *Past. pseudotuberculosis*. Frozen glands, faeces, and throat swabs were cultured for viruses on amnion and MK2 cells—a continuous monkey kidney cell line (Westwood, Macpherson, and Titmuss, 1957).

We were unable to isolate viruses from any of the specimens examined. On the other hand, we were able to find evidence of infection with *Past. pseudotuberculosis* in three of the 12 children from whom both gland and serum were received. There was no serological evidence of infection with the bacterium in the other five patients.

We report here the three cases of pseudotuberculous mesenteric lymphadenitis. As far as we are aware they are the first to be recorded in this country.

Case Reports

Case 1.—R. Sturgeon, a boy aged 10, was admitted to the Leicester Royal Infirmary on May 11, 1959, with a five-day history of central abdominal pain, described as a dull ache. He had had similar attacks previously, but never one so long or so severe as the present one. He felt sick during the illness but did not vomit. He also had mild diarrhoea of two days' duration.

On examination the temperature was 99.8° and the pulse rate 88. There was tenderness and guarding in the right iliac fossa. The rebound sign was equivocal. No masses were felt in the abdomen nor was anything abnormal detected per rectum. The absence of vomiting made the diagnosis somewhat uncertain, but since there appeared to be more tenderness in the right iliac fossa than in the left an appendicectomy was performed.

Operation (May 11).—A normal retrocaecal appendix was present. There was no Meckel's diverticulum. The mesenteric glands were enlarged. The appendix and a mesenteric gland were removed for examination.

Progress.—The temperature became normal on the first post-operative morning. On the second day the patient was restless and irritable, but eventually settled down. No diarrhoea occurred after the operation.

Laboratory Findings.—The mesenteric gland was about ¼ in. in diameter. No organisms were seen in the direct smears, and culture was sterile. Histological examination showed early features of *Pasteurella pseudotuberculosis* infection. Serum taken on May 12, the day after operation, agglutinated *Pasteurella pseudotuberculosis* Types IA and IB to a titre of 1:300. Serum taken five months later showed no agglutinins for *Pasteurella pseudotuberculosis*.

Case 2.—K. Schofield, a girl aged 15, was admitted to the Infirmary on May 13, 1959, with abdominal pain of three days' duration. The pain, which remained localized in the umbilical region, was dragging in nature. There was no nausea or vomiting. Appetite had been poor for three days. The patient complained of slight frequency with some suprapubic pain on micturition. She had been constipated for a week before admission. A similar attack three months previously had passed off in one week.

On examination the temperature was 102.4°, the pulse rate 112, and respirations 20. She did not look ill. The tongue was clean. The abdomen was soft with no guarding. There was tenderness on the left side and right hypochondrium. The descending colon was loaded. Bowel sounds were normal. Urine, S.G. 1012, was acid, straw-coloured, without albumin, sugar, or acetone. A provisional diagnosis of mesenteric adenitis or subacute appendicitis was made.

Operation (May 13).—The appendix was mildly inflamed and not adherent. The pelvis was normal. There was no Meckel's diverticulum. The terminal ileal glands were enlarged. Appendicectomy and gland biopsy were performed.

Progress.—On the day following operation the temperature remained at 100°. It fell to normal on the morning of the 15th, but rose to 100° that evening. The following evening it shot up to 102°. On the evening of the 18th it was 101° and then it fell to subnormal, and remained thus until discharge on May 20. On the day of the high fever the scar was tender, but this settled in 24 hours.

Laboratory Findings.—A direct Gram-stained smear of the gland revealed numerous Gram-negative coccobacilli. Culture yielded a profuse growth of *Pasteurella pseudotuberculosis* (Schofield strain). Serum taken on May 13 agglutinated the homologous organism and *Pasteurella pseudotuberculosis* Types IA and IB to a titre of 1:500. A second specimen of serum taken on October 17 showed no trace of agglutinins. Histological examination of the gland was not made in this case.

Case 3.—G. Burke, a boy aged 12, was sent to hospital by his practitioner on October 19, 1959, with the provisional diagnosis of mesenteric adenitis. He had a two-day history of central abdominal pain, which moved over to the right side on the day before admission. He vomited once on the night of October 18. Bowel movements and micturition were normal. He had slight nausea and anorexia. He had had no previous attacks.

On examination temperature was 101.6° and pulse rate 120. The tongue was slightly coated and moist, and foetor was present. The abdomen was not distended, but tender with muscle guarding in the right iliac fossa. There was no rebound tenderness. No masses were felt. Rectal examination revealed tenderness in the right side over the peritoneal surface. A diagnosis of acute appendicitis was made.

Operation (October 19).—The appendix looked normal. There was no Meckel's diverticulum. Appendicectomy was performed and a very large gland in the ileocaecal angle was removed.

Progress.—Apart from a slight pyrexia of 99° on October 22 the boy made an uneventful recovery.

Laboratory Findings.—No organisms were seen in the direct smears of the gland. Culture yielded *Pasteurella pseudotuberculosis*, and the organism was also isolated from a guinea-pig inoculated with a suspension of the gland (Burke strain). Histological examination showed the characteristic features of *Pasteurella pseudotuberculosis* infection. Serum taken on October 19 agglutinated the homologous organism to a titre of 1:5,000 and *Pasteurella pseudotuberculosis* Types IA and IB to a titre of 1:3,000. Opportunity was taken to study the decline in agglutinins in this patient and the results are described later in the section on serology.

Bacteriology

Examination of Mesenteric Glands.—Each gland was examined as follows: Smears were stained by Gram's method and by a modified acid-fast technique recommended by Cook (1952) for the recognition of *Pasteurella pseudotuberculosis* in tissues. The gland from Case 2 was the only one to show Gram-negative and acid-fast bacilli in the smears.

The gland was then ground up in 5 ml. digest broth, and loopfuls of the suspension were inoculated on 5% horse-blood digest agar and into digest broth. Duplicate cultures were incubated at 22° C. and 37° C. for 48 hours. The Schofield strain was obtained on direct culture at 37° C. On the other hand, Burke's strain was isolated only after preliminary incubation in broth at 22° C. for 24 hours.

Guinea-pig inoculation was carried out only with Burke's gland suspension. The animal became ill on the seventeenth day after inoculation and was killed on the twentieth day. At necropsy there was a large caseating abscess at the site of injection, caseous enlargement of the regional gland, and generalized septicaemic spread with multiple nodules in the liver, spleen, and lungs. *Pasteurella pseudotuberculosis* was isolated in large numbers from the local lesion, organs, and heart blood.

Examination of the Appendix.—The interior of each appendix was exposed and inoculated on blood agar plates, which were incubated at 22° C. and 37° C. and examined after 24 and 48 hours with negative results for pasteurella.

Identification of Schofield and Burke Strains.—Both strains showed similar morphological, cultural, and biochemical characteristics. Both consisted of Gram-negative cocco-bacilli, motile in broth culture at 22° C. and non-motile at 37° C. They grew readily on the usual media, producing on blood agar, after 24 hours at 37° C., flat, dry, non-haemolytic colonies, with crenated edges and a dull granular surface. After 24 hours' incubation at 22° C., small moist colonies with entire edge and smooth shining surface were produced. Colonies grown at 37° tended to agglutinate spontaneously in normal saline, in contrast with those grown at 22° C. which showed no tendency to auto-agglutination. Minute colonies appeared on MacConkey's medium after 24 hours at 37° C. In broth growth was diffuse at 22° C., and tended to be more viscous at 37° C.

Acid without gas was produced after 18 hours at 37° C. in dextrin, glucose, laevulose, maltose, mannitol, rhamnose, and trehalose, after 48 hours in galactose and glycerol, and after four days in salicin. Trace reactions were obtained in sorbitol and xylose. Arabinose, dulcitol, inulin, lactose, raffinose, and sucrose were not fermented after 14 days.

The methyl-red reaction was positive and the Voges-Proskauer reaction negative. Indole and H_2S were not produced and gelatin was not liquefied. Urea was decomposed, litmus milk was rendered alkaline, and nitrates were rapidly reduced. Catalase was present.

Guinea-pigs injected intramuscularly with 0.5 ml. of 24-hour broth cultures of the Schofield and Burke strains died in nine and 18 days respectively. Post-mortem findings were similar to those obtained with Burke's infected gland suspension.

Serology

Strains of seven serotypes and subtypes of *Pasteurella pseudotuberculosis* were obtained from the National Collection of Type Cultures. The antigenic constitution of the different types is shown in Table I.

TABLE I
ANTIGENIC CONSTITUTION OF *PAST. PSEUDOTUBERCULOSIS* ACCORDING TO KNAPP (1956)

Type	Subtype	Somatic Antigen	Flagellar Antigen
I	IA	1 2 3	a
	IB	1 2 4	a
II	IIA	1 5 6	a
	IIB	1 5 7	a
III		1 8	a
IV		1 9	b
V		1 10	a

Living suspensions were used throughout for agglutination tests as recommended by Knapp (1956), who reported that heat-killed (100° C.) antigen was not agglutinated by human antisera. Agglutinating antigen was prepared by growing the organism on tryptose agar at 22° C. After 48 hours the growth was harvested in normal saline, centrifuged, resuspended in saline, agitated with glass beads in a shaker for 20 minutes, and finally diluted to a density equivalent to No. 10 Brown opacity tube. Agglutination tests were carried out in ¼-in. wide round-bottom tubes. One drop of the concentrated suspension was added to 0.5 ml. of each serum dilution and the tubes were incubated for two hours at 37° C., left in the refrigerator overnight, and read

two hours after removal next morning. Parallel tests, using Dreyer's technique and incubating the suspension for 24 hours at 56° C., gave similar results. For absorption, serum diluted 1:10 was poured over the centrifuged deposit of a dense suspension of the absorbing organism and thoroughly mixed. The mixture of serum and bacteria was incubated for two hours at 37° C. followed by 20 hours at refrigerator temperature (+5° C.).

In preparing O antisera it was necessary to take special precautions in order to obtain suitable heat-killed antigens. Many authors have noted the tendency of *Pasteurella pseudotuberculosis* to agglutinate spontaneously in normal saline, especially when the suspension is heated. Recommendations to overcome this difficulty include reduction of salt content to 0.3% (Topping, Watts, and Lillie, 1938), strong shaking of the suspension before boiling (Preston and Maitland, 1952), and the use of cultures grown at 22° C. (Knapp, 1956). By using a combination of these methods we were able to obtain stable heat-killed suspensions. Smooth colonies of a 22° C. culture were sown on tryptose agar and incubated at 22° C. for 24 hours. The growth was harvested in 0.5% saline, agitated with glass beads in a shaker for 20 minutes, washed well in 0.5% saline, shaken again, and then exposed to flowing steam in a Koch sterilizer for two and a half hours or autoclaved at 120° C. for two hours; 0.25% phenol was added as a preservative.

Agglutination Tests with Patients' Sera.—Preliminary slide agglutination tests indicated that agglutinins to Types IA and IB were present in the sera of all three patients. No agglutination was observed with the other serotypes. Because only limited amounts of sera were available tube agglutination tests had to be restricted to reactions with the Burke and Schofield strains and the National Collection of Type Cultures serotypes IA and IB. The results of the agglutination tests with unabsorbed and absorbed sera shown in Table II suggest that the Schofield and Burke strains are antigenically similar and probably belong to Type IA. The results with Sturgeon's serum also indicate infection with *Pasteurella pseudotuberculosis* Type I, but because of the relatively low titres obtained with the unabsorbed serum, we are unable to demonstrate residual titres after absorption with the four antigens.

Typing of Schofield and Burke Strains.—Typing of both strains was confined to the identification of the somatic factors present. Recognition of the flagellar antigen is of little value in the determination of the different types because, with the exception of Type IV, the antigen is common to all members of the group. O agglutinating antisera were prepared in rabbits for Types IA and IB as well as for Schofield and Burke strains. Autoclaved antigen was inoculated intravenously in six doses (0.25 ml., 0.5 ml., 1 ml., 1 ml., 1 ml., 1 ml.) at four-day intervals. The animals were bled on the twenty-eighth day. The results of

TABLE II
RESULTS OF AGGLUTINATION TESTS WITH ABSORBED AND UNABSORBED PATIENTS' SERA

Sera	Agglutination Titres of Sera against Four Strains of *Past. pseudotuberculosis* — Agglutinating Antigens: Schofield	Burke	Type IA	Type IB
Schofield serum				
Unabsorbed	500	500	500	500
Absorbed with				
Schofield strain	<25	<25	<25	<25
Burke strain				
Type IA	25	25	25	
Type IB				
Burke serum				
Unabsorbed	5,000	5,000	3,000	3,000
Absorbed with				
Schofield strain	<25	<25	<25	<25
Burke strain				
Type IA	200	200	200	
Type IB				
Sturgeon serum				
Unabsorbed	300	300	300	300
Absorbed with				
Schofield strain	<25	<25	<25	<25
Burke strain				
Type IA				
Type IB				

TABLE III
RESULTS OF AGGLUTINATION TESTS WITH ABSORBED AND UNABSORBED RABBIT ANTISERA

Sera	Agglutination Titres of Sera against Four Strains of *Past. pseudotuberculosis* — Agglutinating Antigens: Schofield	Burke	Type IA	Type IB
Schofield antiserum				
Unabsorbed	2,000	2,000	2,000	2,000
				trace
Absorbed with				
Schofield strain	<25	<25	<25	<25
Burke strain				
Type IA	200	200	100	
Type IB				
Burke antiserum				
Unabsorbed	5,000	5,000	5,000	5,000
Absorbed with				
Schofield strain	<25	<25	<25	<25
Burke strain				
Type IA	500	500	200	
Type IB				
Type IA antiserum				
Unabsorbed	10,000	10,000	10,000	10,000
Absorbed with				
Schofield strain	<25	<25	<25	<25
Burke strain				
Type IA	500	500	500	
Type IB				
Type IB antiserum				
Unabsorbed	10,000	10,000	5,000	20,000
				trace
Absorbed with				
Schofield strain	<25	<25	<25	2,000
Burke strain				2,000
Type IA				1,000
Type IB				<25

the cross-absorption tests show that the Schofield and Burke strains are antigenically similar to Type IA, thus confirming the findings made with the patients' sera (Table III). Both strains were later sent to Professor Knapp, of Tübingen, who kindly informed us that they belonged to Type IA.

Post-operative Decline in Antibody Titres.—Serum taken from Schofield and Sturgeon five months after operation showed no evidence of agglutinins to *Pasteurella pseudotuberculosis*.

Burke's serum showed a rise in antibody titre two weeks after operation, but thereafter there was a steady decline until agglutinins had all but disappeared five months after onset of the illness (Table IV).

TABLE IV
RESULTS OF AGGLUTINATION TESTS WITH BURKE'S SERA SHOWING DECLINE IN ANTIBODIES

Day after Onset of Illness	Serum Titre
4th	5,000
17th	10,000
33rd	2,000
77th	500
160th	25 trace

Antibiotic Sensitivity Tests.—These were carried out on the Schofield and Burke strains by the flood plate technique using "multodisk" (Oxoid) paper discs. Both cultures were fully sensitive to chloramphenicol (10 μg.), streptomycin (10 μg.), tetracycline (10 μg.), polymyxin B (10 μg.), neomycin (10 μg.), and nitrofurantoin (200 μg.). Resistance was shown to penicillin (1.5 μg.), erythromycin (10 μg.), oleandomycin (5 μg.), novobiocin (5 μg.), bacitracin (5 units), and sulphafurazole (100 μg.).

Histology

Biopsy specimens of appendices and mesenteric glands were fixed in formol saline and embedded in paraffin wax. Sections were stained with haematoxylin and eosin.

Case 1 (Sturgeon).—In the appendix there was no evidence of inflammation. The lymphoid follicles were large with prominent Flemming centres.

In the lymph node there was considerable sinus hyperplasia and the follicles showed well-defined centres. Towards the periphery of the node there was a single small follicle consisting of a group of polymorphonuclear pus cells and swollen cells resembling reticulum cells. The latter were quite large and an occasional cell appeared to have two nuclei. It was a very unusual type of follicle to be seen within a lymph node (Figs. 1 and 2). No organisms could be seen in Gram-stained, Cook-stained, or Ziehl-Neelsen-stained sections.

Case 2 (Schofield).—In this case the lymphoid follicles formed an almost continuous band around the lumen of the appendix and there was a plasma cell infiltration of the mucosa itself. Within the lumen of the appendix there was granular debris, containing fairly large numbers of lymphocytes, and a smear taken from this debris showed a large number of Gram-negative diplobacilli. They were also to be seen within the lymphoid follicles. No lymph node was examined in this case.

Case 3 (Burke).—In the appendix in this case, lymphoid follicles were very prominent and they tended to be two or three layers in depth.

Sinus hyperplasia in the lymph node was distinct. In this instance there were two follicles which appeared to be slightly later in stage than the one observed in Case 1 (Sturgeon). The follicles showed central necrosis with the presence of eosinophilic-staining debris. This necrotic area was infiltrated with plasma cells and polymorphs. It was surrounded by a layer of reticulum-cells showing somewhat haphazard palisading about four cells in depth. Beyond it the reticulum cells showed no orderly arrangement. In this outer zone there was an occasional foreign-body type of giant cell, polymorph and lymphocyte (Figs. 3 and 4). No organisms could be seen in Gram-stained, Cook-stained, or Ziehl-Neelsen-stained sections.

The lesions noted in the lymph glands were not found in the lymphoid follicles of any of the appendices.

Discussion

Until a few years ago human infection with *Pasteurella pseudotuberculosis* was generally regarded as rare. The first authentic case was described by Albrecht (1910). During the period between 1910 and 1952 some 15 cases were reported in the medical literature. Of these, 13 were characterized by a severe typhoid-like illness which terminated fatally in 11 cases.

In contrast to this rare septic type of infection the more frequent benign appendiceal form is now recognized. In addition to numerous cases mentioned in the German literature, other cases have been reported in Hungary (Podhragyai and Fodor, 1956) and in France (Ingelrans, Soots, Poupard, and Vitse, 1957; Girard, Leger, Paulhet, and Duffau, 1959). No cases appear to have been recorded in the English language.

This new disease complex shows a predilection for children and young people (2 to 23 years) and occurs more often in males than in females. The clinical picture is that of acute or subacut

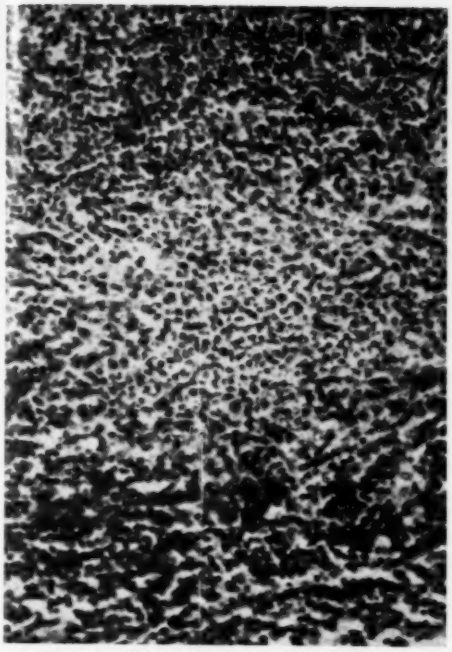

FIG. 1.—Case 1: Follicle consisting of swollen reticulum cells. The centre of the follicle is infiltrated with polymorphs. (Haematoxylin and eosin × 150.)

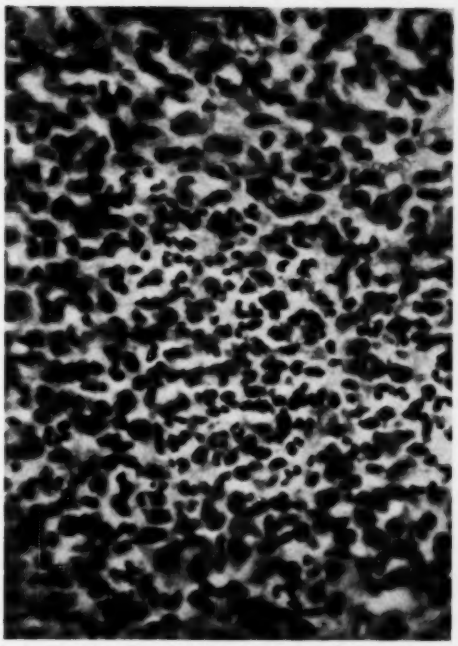

FIG. 2.—Case 1: Centre of follicle. (Haematoxylin and eosin × 425.)

FIG. 3.—Case 3: Follicle showing haphazard palisading of reticulum cells and area of central necrosis infiltrated with polymorphs. (Haematoxylin and eosin × 350.)

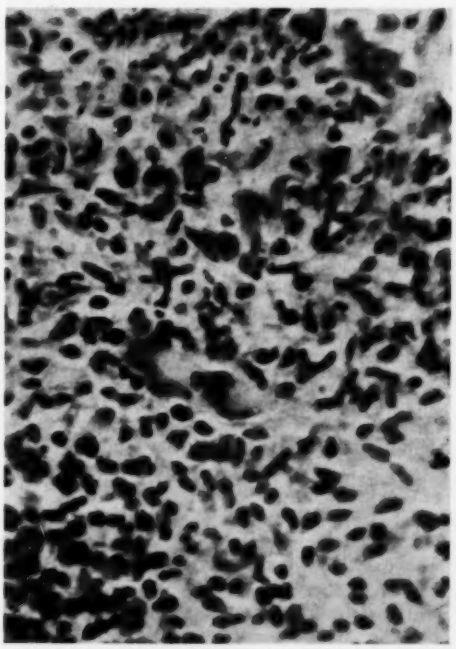

FIG. 4.—Case 3: Foreign-body type of giant cells in marginal zone of reticulum cells. (Haematoxylin and eosin × 350.)

appendicitis with pain in the middle or right lower quadrant of the abdomen. The temperature is elevated to 100° or even higher, and there is invariably a leucocytosis. Laparotomy shows, in most cases, a normal-looking appendix. The lymphatic glands in the ileocaecal angle and in the mesentery are enlarged either singly or in groups. The peritoneum over the lymph nodes is congested and a clear or slightly purulent exudate is often present. Inflammatory infiltration of the terminal ileum has also been described.

In our first two cases there was some preoperative doubt as to the correct diagnosis, but both patients were too unwell to justify withholding an exploratory laparotomy. The third case (Burke) was considered by his own doctor to have mesenteric adenitis, but on admission his symptoms and signs were such that there seemed to be no doubt about the diagnosis of acute appendicitis.

The findings at operation on all three patients were essentially similar. After the first two had been seen—and the diagnosis of pasteurella infection subsequently established—the third case was strongly suspected at operation and later confirmed pathologically. In all three it was obvious that the appendix was not the cause of the trouble ; such exploration as could be carried out through a muscle-splitting incision failed to reveal any abnormality other than in the affected ileocaecal glands. Owing to the limited exposure obtained, the extent of the glandular enlargement could not be determined. Suppuration was not seen in any of the three cases and there was no gross oedema of the mesentery around any of the affected nodes. Peritoneal exudate was not observed.

The naked-eye appearance of the glands suggests that it may be possible to distinguish between those cases due to infection with *Pasteurella pseudotuberculosis* and those due to other causes. The glands seen in the non-pasteurella group appear to be pale, flattened rather than round, and uniformly enlarged. In our three cases associated with pasteurella infection the glands were dark in colour with one or two white streaks across the surface, almost round, and variable in size, the largest being about the size of a cherry and the smallest no bigger than a dried pea. All were firm, not hard, fixed within the mesentery but not adherent to it. Removal was easy and there was no undue tendency to bleeding.

The definitive diagnosis, however, is established by the isolation of the organism from the lymphatic glands, by the demonstration of specific antibodies in the serum, and through histological examination of the lymph nodes.

Pasteurella pseudotuberculosis is not readily isolated from infected glands. Only 12 of the 117 cases mentioned by Knapp, and one of nine cases reported by Schmidt (1959), were confirmed by isolation of the organism from the lymph nodes. That incubation at 37° C. may not be sufficient is shown by the behaviour of Burke's strain, which was isolated only after preliminary incubation in broth at 22° C. Identification of the organism itself is not difficult. Its motility in 18-hour broth cultures at 22° C., its ability to grow on MacConkey medium and to decompose urea, and the caseous nodules produced in guinea-pigs after experimental inoculation, help to distinguish it from other members of the pasteurella group.

Antibodies to *Pasteurella pseudotuberculosis* can be demonstrated in the blood during the acute phase of the illness. By means of agglutination tests with live, smooth variants of *Pasteurella pseudotuberculosis* Types I–V, titres of 1:80 to 1:12,800 have been recorded. In our own cases titres varied from 1:300 on the sixth day of Sturgeon's illness to 1:5,000 on the fourth day of Burke's attack. The tests are specific only for Types I, III, and V. The specificity of the agglutination test for Types II and IV is affected by the antigenic relationships which exist between *Pasteurella pseudotuberculosis* Type II and factor IV of the salmonella B group and *Pasteurella pseudotuberculosis* Type IV and factor IX of the salmonella D group (Kauffmann, 1933 ; Knapp, 1955). These give rise to fairly strong cross-reactions. In order that a serological diagnosis can be made it is necessary to absorb the patient's serum with the corresponding strain of the B or D group. However, such absorptions are not often necessary, since more than 90% of pseudotuberculous infections in man are caused by *Pasteurella pseudotuberculosis* Type I. The specificity of the agglutination test was confirmed by Knapp (1956), who was unable to find antibodies against *Pasteurella pseudotuberculosis* in the blood of 76 children with typical appendicitis. In 1,601 sera obtained for Wassermann and Widal tests he found antibodies to Types I, III, and V in only nine patients, all of whom gave a history suggestive of mesenteric adenitis. If complications do not occur, agglutinins disappear within a few months. All our three cases, who remained well after operation, showed little or no antibody in the blood five months later. In Burke's case there was a rise in titre a fortnight after operation. Two and a half months later the titre had fallen

considerably, and at the end of five months only a trace reaction was obtained with a serum dilution of 1:25.

The histological findings confirm the observation made by Knapp and Masshoff (1954). In our third case, the appearance of the two abnormal follicles consisting of reticulum cells with central necrosis was most striking. The arrangement of the reticulum cells around the periphery of the necrotic area was much more haphazard than one sees in sarcoidosis, and the central eosinophilic tissue did not resemble caseation of tubercle. The same phenomenon was observed in Case 1, but to a lesser degree. These changes are not specific for pseudotuberculosis since they have also been observed in tularaemia, lymphogranuloma inguinale, and cat-scratch fever. Nevertheless, they appear to be a characteristic feature of infection with *Pasteurella pseudotuberculosis* and were noted by Knapp (1959) in 86% of his cases.

The epidemiology of human pseudotuberculosis remains obscure. *Pasteurella pseudotuberculosis* is known to cause epizootics in guinea-pigs and turkeys, and sporadic infections have been reported in rabbits and hares, cattle, chickens, goats and pigs, cats and dogs, pigeons, canaries, sparrows, and blackbirds. It has not been possible to relate human cases to contact with domestic animals or to the use of their products. In the present cases the only history of contact with animals was that a budgerigar was kept by the Burke family and that 12 days before the boy was taken ill the bird sickened and remained off-colour for seven days, when it recovered, apparently without any ill effects. It was not possible to obtain the bird for examination, but droppings from the cage were cultured for *Pasteurella pseudotuberculosis* with negative results.

Little has been reported on the treatment of human pseudotuberculosis. Four cases of the severe septic type of infection were treated with sulphonamides and antibiotics and two of them survived (Knapp, 1959). Although antibiotics have been used for the treatment of the benign appendiceal form and as a prophylactic measure to prevent possible post-operative complications, it is difficult to assess their value, as most of these cases run a benign course, and those treated surgically usually make an uneventful recovery. Should the administration of antibiotics be considered necessary, the results of tests *in vitro* suggest that the broad-spectrum antibiotics would be the drugs of choice.

It is evident from the results of this investigation that pseudotuberculous mesenteric adenitis occurs in this country as well as on the Continent. While the definitive diagnosis rests on the isolation of the organism from the lymphatic glands the possibility of adhesions arising from routine gland biopsy must be taken into consideration (Aird, 1958). In those cases where biopsy is deemed inadvisable, or where provisional diagnosis of acute mesenteric adenitis is made, it may be possible to arrive at a diagnosis in many cases of pasteurella infection by carrying out agglutination tests with the patient's serum against the five serotypes of *Pasteurella pseudotuberculosis*.

The investigation would not have been possible but for the interest of the consultant surgeons at the Leicester Royal Infirmary and the Leicester General Hospital; all kindly agreed to their patients with mesenteric adenitis being included in the survey. For access to the records of Cases 1 and 2 we are indebted to Mr. D. McGavin, and for Case 3 to Mr. E. R. Frizelle.

Dr. E. Milford Ward's observations on the histological findings were of considerable value in confirming the diagnoses. We are especially grateful to Mr. B. J. Fowler for his interest and advice.

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FOLIC ACID AND OTHER ABSORPTION TESTS IN LYMPHOSARCOMA, CHRONIC LYMPHOCYTIC LEUKAEMIA, AND SOME RELATED CONDITIONS

BY

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An improved folic acid absorption test is described in detail.

Using the folic acid and other absorption tests, a study of intestinal function has been made in eight patients with lymphosarcoma, eight with chronic lymphocytic leukaemia, two with Hodgkin's disease, and one each with reticulum cell sarcoma, Whipple's disease, and macroglobulinaemia. It was found that laboratory evidence of intestinal malabsorption was extremely common in patients with this group of diseases.

The folic acid absorption test was the most sensitive single index of malabsorption of the tests used in this study.

Correlation between the different absorption tests was poor, and there does not appear to be a characteristic biochemical profile of intestinal function in these patients.

The pathology and clinical features of primary lymphosarcoma and allied tumours of the alimentary tract have been described by several authors, including Spellberg and Zivin (1949) and Skrimshire (1955). These tumours are uncommon, but may produce dyspepsia, gastrointestinal haemorrhage, or intestinal obstruction. A rare complication is the development of steatorrhoea and overt malabsorption. This was first described by Fairley and Mackie (1937), and the relevant literature has recently been reviewed by Sleisenger, Almy, and Barr (1953), who reported four further cases.

In the majority of patients with lymphosarcoma, involvement of the gastrointestinal tract and mesenteric lymph nodes is an incidental pathological finding associated with infiltration of many other tissues. It rarely produces significant symptoms, and is not regarded as important in the natural history of the disease. In a recent patient (As) who underwent splenectomy for acquired haemolytic anaemia associated with lymphosarcoma, we were impressed by the appearance of stiffened oedematous bowel at operation, although the patient had noted only slight symptoms of intestinal dysfunction. We therefore decided to investigate intestinal function in an unselected series of patients with lymphosarcoma not arising primarily in the alimentary tract. Since somewhat similar infiltration of the intestinal wall and mesenteric lymph nodes may also occur in patients with chronic lymphocytic leukaemia, patients with this condition were included in the investigation.

Special emphasis has been given to the folic acid absorption test, as Chanarin, Mollin, and Anderson (1958) reported that injected folic acid was cleared from the plasma more rapidly than normal in some patients with leukaemia and related disorders. These patients had an associated megaloblastic anaemia, and it was considered that the anaemia was due to folic acid deficiency. Proliferating neoplastic cells have greater than normal requirements for folic acid (Swendseid, Bethell, and Bird, 1951); however, Chanarin and his colleagues noted that some patients failed to absorb folic acid normally.

The present paper describes in detail the folic acid absorption test used in this laboratory, and reports the results of several intestinal absorption tests in patients with lymphosarcoma and related conditions, comparing these to folic acid absorption and the clinical state of the patient.

Methods

Folic Acid Absorption Test.—The folic acid absorption test was performed as follows.

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Preparation of the Subject.—The subjects were loaded with folic acid before the test. This was achieved in normal control individuals by giving 15 mg. folic acid orally on each of the fourth, third, and second days before the test. In patients in whom the absorption of folic acid was to be studied, 15 mg. sodium folate (" folvite," Lederle) was given by subcutaneous injection on each of these days. No folic acid therapy was given the day before the test. Subjects fasted from nine hours before until after the completion of the test. During this time water was allowed ; food and liquids other than water may affect serum turbidity readings and were withheld.

Test Procedure.—Five millilitres of venous blood were withdrawn from the fasting subject and allowed to clot in a sterile tube at 37° C. A solution of sodium folate in 20 ml. of water containing the equivalent of 2 mg. folic acid was then drunk. This solution was prepared from an ampoule of sodium folate containing the equivalent of 15 mg. folic acid/ml.

TABLE I

CYTOLOGICAL, HAEMATOLOGICAL, AND CLINICAL FINDINGS

Patient	Age	Sex	Cytological Diagnosis	Haemoglobin (g./100 ml.)	Total Leucocytes (thousands/c.mm.)	Differential White Blood Count (%)					Clinical Features
						P	L	M	Abn.	Other	
As	58	M	Lymphosarcoma	6·5	4·4	37	58	2	—	3	Weight loss. Haemolytic anaemia corrected by splenectomy. Occasional diarrhoea
Bo	54	M	,,	15·2	5·3	57	31	11	—	1	Weight loss. Generalized lymphadenopathy including abdominal masses. Anorexia, vomiting, abdominal pain
Co	68	M	,,	11·3	19·0	26	63	3	7	1	Lymphadenopathy and hepatosplenomegaly. No gastrointestinal symptoms
Ev	63	F	,,	9·1	2·2	49	31	14	—	6	Weight loss. Lymphadenopathy and hepatosplenomegaly. Abdominal masses. Occasional diarrhoea. Ascites and pleural effusion
Fr	72	M	,,	9·1	6·0	20	66	10	4	—	Weight loss. Splenomegaly. Recurrent melaenal diarrhoea. Staphylococcal skin infections
Mp	50	M	,,	12·5	8·4	41	46	6	7	—	Weight loss. Lymphadenopathy and splenomegaly. No gastrointestinal symptoms. Recurrent infections
Mk	42	F	,,	12·6	1·5	50	24	26	—	—	Weight loss. Abdominal and mediastinal lymphadenopathy. No gastrointestinal symptoms. Pleural effusion
Mo	62	F	,,	10·1	3·5	66	24	10	—	—	Weight loss. Hepatosplenomegaly. Abdominal distension with colicky pain and recurrent melaenal diarrhoea. Osteoporosis and vertebral collapse
Ba	84	M	Chronic lymphocytic leukaemia	13·1	129	1	96	3	—	—	Weight loss. Massive lymphadenopathy inguinal region with oedema of leg. No gastrointestinal symptoms
Be	66	M	,, ,,	15·2	92	1	97	1	—	1	Weight loss. Generalized lymphadenopathy. Splenomegaly. Abdominal masses. No gastrointestinal symptoms
Bl	38	M	,, ,,	9·1	600	2	98	—	Occas.	—	Weight loss. Generalized lymphadenopathy. Hepatosplenomegaly. Terminal melaenal diarrhoea. Weakness
Go	72	M	,, ,,	11·1	129	3	97	—	—	—	Weight loss. Generalized lymphadenopathy. Hepatosplenomegaly. Abdominal distension and flatulence
Md	65	M	,, ,,	11·1	211	1	99	—	Occas.	—	Weight loss. Generalized lymphadenopathy. Hepatosplenomegaly. Occasional diarrhoea. Recurrent infections
Ca	68	F	,, ,,	10·3	16·0	8	91	1	—	—	Generalized lymphadenopathy. Hepatosplenomegaly. No gastrointestinal symptoms. Recurrent infections
Ho	75	F	,, ,,	10·9	84	9	91	—	—	—	Weight loss. Splenomegaly. Recurrent diarrhoea. Recurrent infections
Cr	79	F	,, ,,	11·5	137	3	97	—	—	—	Splenomegaly. No gastrointestinal symptoms
Ct	21	M	Hodgkin's disease	10·1	12·2	79	8	10	—	3	Weight loss. Abdominal masses. Nausea and vomiting following radiotherapy. Recurrent infections
Kr	50	F	,,	12·2	6·4	83	8	8	—	1	Weight loss. Abdominal masses. No gastrointestinal symptoms, except diarrhoea following radiotherapy
We	34	M	Reticulum cell sarcoma	8·9	1·9	12	59	12	17	—	Splenomegaly. Infiltration stomach, ileum, large bowel at necropsy. Diarrhoea. Anaemia
Do	54	M	Whipple's disease	11·3	12·0	94	4	2	—	—	Weight loss. Diarrhoea. Abdominal distension. Pigmentation
Wo	74	F	Macroglobulinaemia	9·1	6·6	53	37	8	—	2	Weight loss. Abdominal pain of ulcer type. Achlorhydria. Recurrent infections. Anaemia. Previous nephrectomy

TABLE II
OTHER LABORATORY AND RADIOLOGICAL FINDINGS

Patient	Total Serum Protein	Serum Albumin	Serum Globulins	Plasma Carotene	Daily Faecal Fat Excretion	Percentage Fat in Faeces	Xylose Absorption	Folic Acid Absorption	^{58}Co-B_{12} Absorption	Radiological Findings
Normal values	6·0–7·5 g./100 ml.	3·5–5·5 g./100 ml.	1·5–3·5 g./100 ml.	Over 70 μg./100 ml.	Less than 5 g./Day	Less than 25%	Greater than 4 g. in 5-Hour Urine	Serum Peak 70–130 μmg./ml.	Urinary Excretion 16–36%	
As	6·2	3·3	2·9	—	6·7	—	—	48	—	Stomach normal
Bo	6·4	2·9	3·5	41	—	—	6·9	65	—	Mucosal thickening of stomach. Normal jejunal pattern
Co	6·6	3·1	3·5	157	—	10	—	60	19·2	—
Ev	4·5	2·2	2·3	53	—	62	2·5	10	19·3	—
Fr	6·8	2·3	4·5	60	—	27	5·9	28	26·3	Duodenal diverticulum. Abnormal jejunal pattern
Mp	6·0	2·2	3·8	16	—	32	5·7	18	—	—
Mk	6·4	2·7	3·7	27	—	—	6·3	82	4·7	—
Mo	7·9	1·8	6·1	27	1·9	6·4	2·6	38	14·4	Normal stomach and jejunal pattern. Gross osteoporosis
Ba	5·6	2·2	3·4	83	—	—	2·5	20	—	—
Be	—	—	—	—	—	—	—	12	—	Normal stomach and jejunal pattern
Bl	5·9	3·6	2·3	—	—	—	—	24	—	—
Go	6·9	4·4	2·5	56	—	36	2·9	66	—	Normal stomach and jejunal pattern
Md	5·3	2·6	2·9	42	6·2	49	2·1	20	6·7	Normal jejunal pattern
Ca	5·7	3·2	2·5	52	—	9	5·7	182	—	Normal stomach and jejunal pattern
Ho	6·3	3·0	3·3	24	—	7·2	Incontinent	32	—	—
Cr	6·9	2·9	4·0	123	—	15	2·3	116	24·1	—
Ct	8·0	2·9	5·1	22	—	25·2	5·3	44	—	Normal stomach
Kr	6·9	3·4	3·5	33	—	28	4·8	35	4·5	—
We*	—	—	—	—	—	—	—	8	—	—
Do	5·0	2·3	2·7	3	16	—	2·1	52	Normal†	Abnormal jejunal pattern
Wo	6·9	2·6	4·3	155	—	—	3·8	180	28·6	Normal sto mach and jejunal pattern

* This patient had been receiving oral folic acid therapy for a month before the test. †Faecal excretion test used. 0·68 μg. of a 1 μg. oral dose ^{58}Co-B_{12} was absorbed.

The folic acid content of an ampoule of the same batch number had been checked previously by assaying against a preparation of crystalline folic acid (Light) stated to be at least 99% pure. Blood samples were withdrawn at hourly intervals for three hours. Serum from all four blood samples was stored at −20° C. until tested for folic acid content.

Assay of Folic Acid.—Serum folic acid activity was estimated microbiologically using a mutant strain of *Streptococcus lactis* R (A.T.C.C. 8043). The mutant strain is approximately three times as sensitive to folic acid as the standard strain. The technique used was essentially that of Teply and Elvehjem (1945). Growth turbidity was recorded with an EEL nephelometer.

Other Tests Used.—Other tests included radioactive vitamin B_{12} absorption (Pitney and Stokes, 1958), xylose absorption (Benson, Culver, Ragland, Jones, Drummey, and Bougas, 1957), and estimations of faecal fat, serum proteins and plasma carotene by standard methods. Fat balances were estimated by the method of Frazer (1956). In some patients radiological examination of the stomach and small bowel was also performed. In a few patients estimations of serum calcium and inorganic phosphate were obtained.

Subjects Studied

Folic acid absorption tests were carried out on 19 normal subjects.

A combination of tests, including the folic acid test in all instances, was performed on eight patients suffering from lymphosarcoma, eight suffering from chronic lymphocytic leukaemia, and five with miscellaneous related conditions. Brief clinical and haematological notes on these patients are included in Table I, and the relevant laboratory results in Table II.

The diagnosis of lymphosarcoma was proved in all cases by histological examination of biopsy material. In five patients the peripheral blood was free of abnormal cells; three showed some abnormal cells, but the appearances were not those of lymphocytic leukaemia. One patient (As) had significant anaemia, which was due to splenic hypersequestration and responded to splenectomy. Clinically, seven of the eight had recent weight loss, and five symptoms referable to the gastrointestinal tract. In one (Bo) these consisted of anorexia and pain suggestive of peptic ulcer. However, he had no diarrhoea or intestinal colic. Two (As and Ev) had mild, transitory bouts of diarrhoea, and in a third (Fr) there was a past history of recurrent melaena. In one patient only

(Mo) were bowel symptoms prominent; she had repeated episodes of diarrhoea with melaena requiring blood transfusion.

The eight patients with chronic lymphocytic leukaemia all had blood counts typical of this condition (Table I). Six had recently lost weight, two (Md and Ho) had mild transient diarrhoea, and one (Go) abdominal distension with flatulence and vague abdominal pain. Melaena was present in one patient (Bl) who developed terminal melaenal diarrhoea.

The five patients with miscellaneous related conditions comprised two patients suffering from Hodgkin's disease, and one each with reticulum cell sarcoma, Whipple's disease, and macroglobulinaemia. Both patients with Hodgkin's disease had recent weight loss, but no gastrointestinal symptoms except transient nausea and vomiting in one and diarrhoea in the other following radiotherapy. Diarrhoea was a prominent symptom of the patient with reticulum cell sarcoma. The patient with Whipple's disease is to be reported in full elsewhere (Maddocks, Ralston, and Weiden, 1960). This patient had clinical malabsorption and steatorrhoea. The diagnosis was established by peroral suction biopsy of the small bowel. The patient with macroglobulinaemia has been included in this series as this condition is thought by some to represent a variant of lymphosarcoma, although she had no clinical signs of this disease. Details of this patient have been published previously (Pitney, O'Sullivan, and Owen, 1958).

No patient in any group had clinical or radiological evidence of primary intestinal tumour, although some had palpable masses due to enlarged mesenteric lymph nodes. One patient (Bo) had radiographic appearances suggestive of gastric lymphosarcoma, but these appeared late in his disease, and there were no signs of small bowel infiltration. None of the patients had associated megaloblastic anaemia. They were studied at a random time during the course of their disease, but wherever possible when they were not receiving active treatment. Some had, however, received prior treatment with nitrogen mustard, "chlorambucil," or radiotherapy.

Finally, folic acid absorption tests were also carried out on four patients suffering from extensive metastatic carcinoma of non-alimentary origin. These patients were not studied fully, and are not discussed in detail below.

Results

(1) **Folic Acid Absorption Tests.**—The results are as follows:

Normal Subjects. — Measurable folic acid activity could be demonstrated in every serum sample obtained from 19 normal adults for three hours after an oral dose of sodium folate containing the equivalent of 2 mg. folic acid. In 17, the peak serum concentration was found at the end of the first hour. In the remaining two subjects, the peak occurred at the second hour. Serum concentrations were falling rapidly by the third hour.

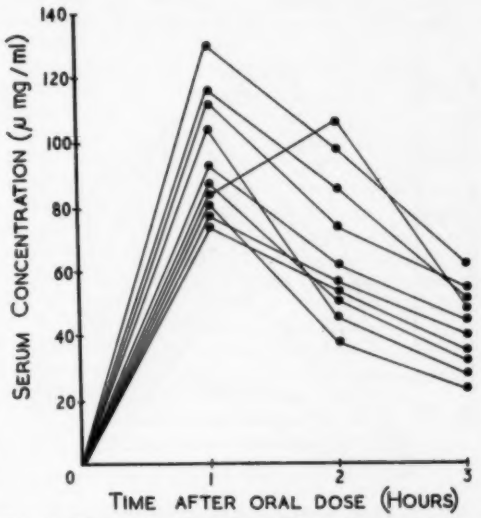

FIG. 1.—Serum folic acid concentrations in 10 normal subjects following a standard oral dose of sodium folate containing the equivalent of 2 mg. folic acid.

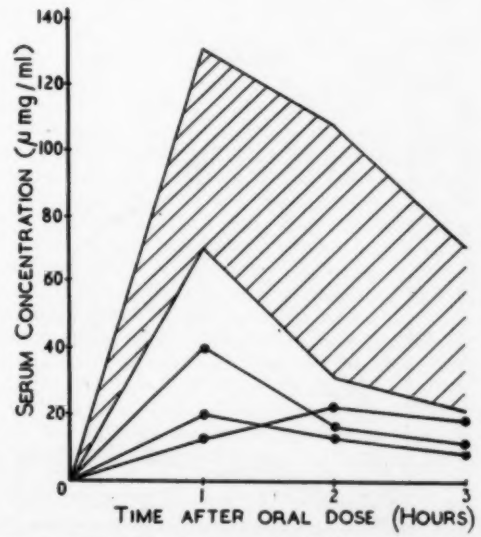

FIG. 2.—Serum folic acid concentrations in three normal subjects who received one half the standard dose. The shaded area represents the normal range of values obtained with the standard oral dose.

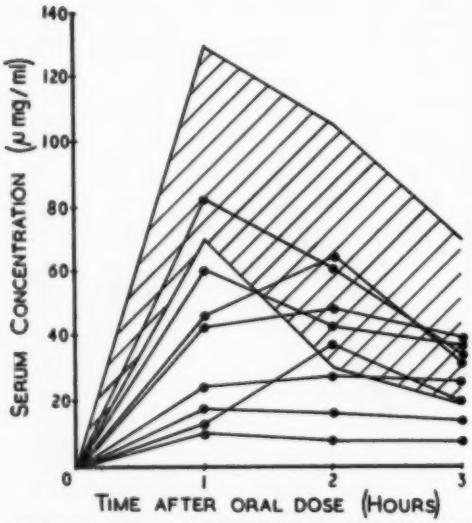

FIG. 3.—Serum folic acid concentrations in eight patients with lymphosarcoma.

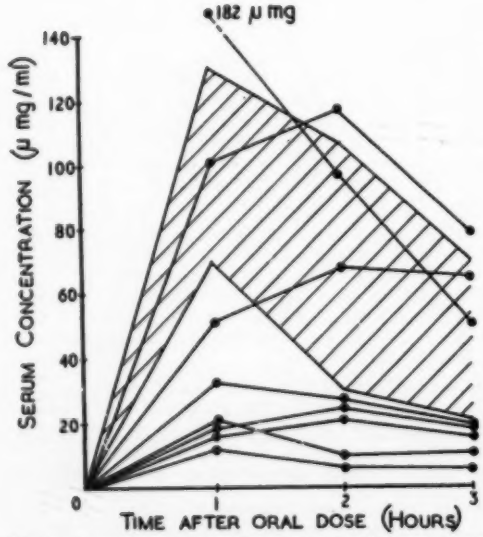

FIG. 4.—Serum folic acid concentrations in eight patients with chronic lymphocytic leukaemia.

The peak concentrations attained ranged from 70 to 130 μmg. folic acid per millilitre of serum. Fig. 1 shows a selection of 10 normal folic acid absorption curves which are representative of the group. This selection includes the highest and lowest curves obtained in the 19 normal subjects.

Measurable serum folic acid activity was obtained in normal subjects after 1 mg. orally, but the curves were flatter (Fig. 2). The three subjects shown in this figure were tested subsequently with the 2 mg. dose and the absorption curves fell within the limits shown in Fig. 1. After these experiments, a standard dose of sodium folate containing the equivalent of 2 mg. folic acid was used in all instances. A peak serum concentration less than 60 μmg./ml. was considered abnormal.

Patients with Lymphosarcoma.—In Fig. 3 are shown the absorption curves given by the eight patients suffering from lymphosarcoma. Five of the eight patients showed subnormal peak serum folic acid concentrations. Four of the five patients with possible small bowel involvement on clinical grounds showed subnormal peak concentrations, as did five of the seven with recent significant weight loss.

Patients with Chronic Lymphocytic Leukaemia.—The absorption curves of the eight patients with chronic lymphocytic leukaemia are shown in Fig. 4. Five showed subnormal peak serum concentrations. These five all had significant weight loss. Three of the four patients with symptoms suggestive of small bowel involvement produced abnormal curves. One patient (Ca) had an abnormally high peak serum concentration. She was receiving treatment for a urinary tract infection at the time of investigation. This is discussed more fully below.

Patients with Miscellaneous Conditions.—The absorption curves of these patients are shown in Fig. 5. The two patients with Hodgkin's disease both showed subnormal peak serum concentrations. One of these patients (Kr) was, however, receiving radiotherapy to the abdomen at the time of the test for para-aortic lymphadenopathy and had temporary diarrhoea. Both were losing weight steadily. The absorption curve of patient We was very flat with a peak serum concentration of 8 μmg./ml. This patient had moderate diarrhoea and post-mortem examination later showed extensive infiltration of the bowel with neoplastic cells. The patient with Whipple's disease (Do) also showed a subnormal peak serum concentration. He had steatorrhoea and a true clinical malabsorption syndrome. The

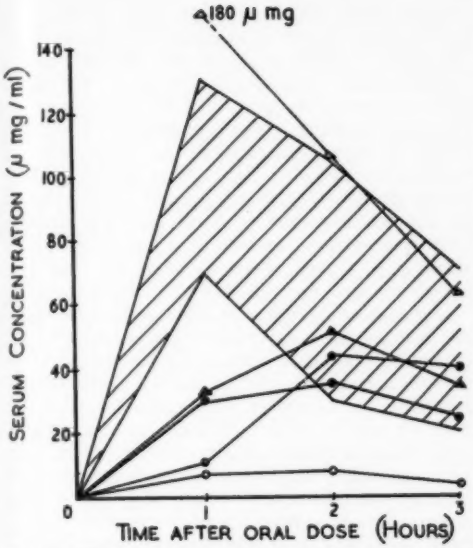

FIG. 5.—Serum folic acid concentrations in patients with Hodgkin's disease (●—●), reticulum cell sarcoma (○—○), Whipple's disease (▲—▲), and macroglobulinaemia (Δ—Δ).

patient with macroglobulinaemia (Wo) had an abnormally high peak serum concentration. It is of interest that she had undergone nephrectomy some years previously.

The four patients with disseminated carcinoma all showed peak serum folic acid concentrations within the normal range.

(2) **Other Investigations.**—Results of the other investigations suggested frequent gastrointestinal involvement in the patients studied.

The serum albumin concentration was below 3.5 g. per 100 ml. in 17 of 19 patients studied, and below 3.0 g. per 100 ml. in 12 of these. All patients with lymphosarcoma had some hypoalbuminaemia, as did five of the seven with chronic lymphocytic leukaemia. No patient in any group revealed hypoglobulinaemia, but increased serum globulin was present in four of eight with lymphosarcoma, and one patient each with chronic lymphocytic leukaemia, Hodgkin's disease, and macroglobulinaemia. This frequent occurrence of hyperglobulinaemia meant that, despite the frequent low serum albumin concentrations, the total serum protein levels were below 6.0 g. per 100 ml. in only six of 19 patients, four of whom were suffering from chronic lymphocytic leukaemia. Statistical analysis showed no significant relation between serum albumin or globulin levels and low peak serum folic acid concentrations, but the association between hypoproteinaemia and diminished folic acid absorption was significant at the 5% level.

Diminished fat absorption was suggested in 13 of 17 patients by low plasma carotene concentrations (less than 70 μg. per 100 ml.), and biochemical steatorrhoea was shown in seven of 12 by increased faecal fat contents in random faecal specimens. Complete six-day fat balances confirmed steatorrhoea in three of four patients. The patient with a normal fat balance (Mo) was, however, unable to tolerate a diet containing 50 g. fat daily, and this result must therefore be considered equivocal. The three groups of patients did not differ significantly in these results, nor was there significant correlation between either low plasma carotene levels or high faecal fat contents and low peak serum folic acid concentrations.

Xylose absorption tests were performed on 15 patients, and impaired carbohydrate absorption demonstrated in eight. The results showed some differences in the three groups of patients. Only two of six with lymphosarcoma had abnormal xylose absorption tests, while abnormal xylose tests were shown in four of five with chronic lymphocytic leukaemia. There was again no significant correlation between xylose absorption and peak serum folic acid concentrations.

The other investigations may be mentioned briefly. Four of 10 patients investigated were found to have defective ^{58}Co–B_{12} absorption. The radioactive vitamin B_{12} was given orally without intrinsic factor and the defective absorption could have been due to either deficiency of endogenous intrinsic factor secretion or to defective absorption of the vitamin B_{12}–intrinsic factor complex. Only one patient of seven studied (Do, with Whipple's disease) had hypocalcaemia. Radiological studies suggested lymphosarcomatous infiltration of the stomach in one patient (Bo), although he had a normal small bowel pattern. The typical segmentation pattern of the malabsorption syndrome was seen in only two of nine patients, one with lymphosarcoma and one with Whipple's disease.

Comparison of the folic acid absorption test with the other biochemical parameters used is difficult, for in these patients, as is usually the case in studies of intestinal absorption, the biochemical profiles varied in apparently random fashion from patient to patient. In general terms, however, the folic acid absorption test appeared to be the most sensitive of the methods used in this study, and in all patients with low peak serum folic acid concentrations collateral evidence of malabsorption was obtained, and such patients tended to show

evidence of multiple absorptive defects. Thus, all patients, except Ca, with a total serum protein concentration below 6.0 g. per 100 ml. had abnormal folic acid absorption tests. Of the 21 patients studied in detail, low peak serum folic acid concentrations were observed in 14, while hypoproteinaemia was found in six of 19, biochemical steatorrhoea in nine of 14, and depressed xylose absorption in eight of 15. Low plasma carotene levels were frequent, but difficult to interpret in patients who were frequently anorexic.

Discussion

The folic acid absorption test described in this paper differs in some respects from that reported by Chanarin, Anderson, and Mollin (1958). We consider it important to fast the patient in the strict sense. Even the amount of milk contained in a cup of tea may alter the turbidity of serum as recorded by a nephelometer, and the usual light breakfast containing buttered toast may cause naked-eye turbidity of serum. The inclusion of a preliminary serum blank in the assay of test serum samples allows correction to be made for non-specific turbidity. Chanarin and co-workers used a test dose of crystalline folic acid dissolved in dilute alkali. Folic acid is likely to be precipitated out of this solution in the acid milieu of the gastric contents. "Folvite" appears to be more stable at low *p*H and is probably absorbed more rapidly. This may explain why 17 of the 19 normal subjects reported in this paper showed peak serum levels after the first hour, whereas the controls of the workers mentioned above showed peaks mostly at the second hour. A 2 mg. dose of folic acid as "folvite" gave similar serum values to those described by Chanarin *et al.* using a 3 mg. dose of folic acid dissolved in dilute alkali. A 2 mg. oral dose gave more satisfactory curves than a 1 mg. dose (Fig. 2).

The serum concentration of folic acid in these tests is influenced by the amount of folic acid absorbed from the intestine during the time of testing, the rate of deviation to the tissues and the rate of renal excretion of folic acid. In renal failure delayed excretion of folic acid might be expected and we have found abnormally high peak serum concentrations in patients with uraemia. Patient Ca (Fig. 4) was suffering from pyelonephritis at the time the test was performed, and patient Wo (Fig. 5) had undergone a previous nephrectomy. Coexisting renal disease is probably a contraindication to this test.

The possibility has been considered that the subnormal folic acid absorption curves observed in this investigation were due to increased tissue requirements for folic acid rather than defective absorption. Pollack, Taylor, and Williams (1942) found higher amounts of folic acid in various types of neoplastic tissue than in the corresponding normal tissue, and Swendseid *et al.* (1951) found higher concentrations of folic acid in leukaemic leucocytes than in normal white cells. Increased tissue requirements does not seem a satisfactory explanation since the four patients with disseminated carcinoma tested presumably also had increased tissue demands for folic acid but all showed normal absorption curves.

Inadequate loading with folic acid before the absorption tests is an unlikely explanation for the subnormal curves. Urinary excretion of folic acid was measured in two normal subjects who each received injections of 15 mg. folic acid on three successive days. This is the loading procedure used in preparation of patients for the tests. Total three-day urinary excretion values in these normal subjects were 17.9 and 19.3 mg. respectively. Assuming that biliary or intestinal loss of folic acid was not significant, these subjects retained more than one half of the total 45 mg. folic acid injected. The highest concentration of total apparent folic acid activity in the body is stated to be in the liver and the concentration in this organ is normally in the range from 1.3 to 5.8 μg./g. wet tissue (Girdwood, 1952, 1953). A normal liver thus contains no more than 10 mg. material with folic acid activity. The loading injections would be expected to more than double the normal body stores.

Finally, the results of other absorption tests in these patients support the view that the low folic acid curves were indeed the result of impaired intestinal absorption, and suggest further that the folic acid absorption test may be the most sensitive single test of intestinal absorption, at least in this group of patients. It would therefore appear that unselected patients suffering from lymphosarcoma and chronic lymphocytic leukaemia commonly show laboratory evidence of intestinal malabsorption. In the majority of our patients there were no clinical signs of severe gastrointestinal involvement. Certainly the typical sprue syndrome was seen in only one patient. Malabsorption could explain some of the puzzling clinical features associated with terminal lymphosarcoma. Ascites and oedema of the lower extremities are often ascribed to pressure from enlarged abdominal lymph nodes. In these patients hypoproteinaemia is common and impaired protein absorption or exudative enteropathy (Gordon, 1959) might offer an alternative mechanism. We have found that

such patients are often improved after intravenous therapy with salt-poor concentrated serum albumin. Weight loss, which is often a prominent feature in both lymphosarcoma and chronic lymphocytic leukaemia, is also common with intestinal malabsorption. The occasional development of megaloblastic anaemia in these patients could be due to malabsorption of folic acid as well as to the increased demands of proliferating neoplastic tissue for folic acid.

We wish to thank Sister A. Smith and Dr. M. F. Carruthers for their help in the preparation of the patients, and Mr. R. E. Davis and Mr. P. Onesti for technical assistance with the microbiological assays. Dr. D. H. Curnow carried out the biochemical estimations.

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ANTI-GAMMA-GLOBULIN CONSUMPTION TEST IN HASHIMOTO'S DISEASE

BY

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A simple anti-gamma-globulin consumption test for use in suspected Hashimoto's disease is described. Results in cases with positive and negative tanned red-cell tests are described.

The anti-gamma-globulin consumption test was introduced by Steffen (1954, 1955) as a means of demonstrating either circulating or sessile antibody. Its application to the study of leucocyte and platelet antibodies and of auto-antibodies in rheumatic fever and arthritis has been reported, chiefly in the German literature; recently, Steffen (1960) has summarized its uses in immuno-haematology.

The present investigation was made with a closely similar method differing only in minor technical detail from that elaborated by Steffen and his colleagues. Hashimoto's disease has been used as a fairly well worked-out model of auto-immune disease, because an initial attempt to apply the method to the study of multiple sclerosis encountered certain difficulties.

In principle, the method is that of Coons' fluorescent microscopy (Coons, 1956) carried out in a test-tube. So far, we have applied it only to the demonstration of circulating antibody (indirect test of Steffen, 1960) since suitable biopsy specimens have not been available for the investigation of sessile antibodies (direct test).

To demonstrate circulating antibody capable of becoming fixed to thyroid tissue, the serum under test is brought into contact with a suitable finely dispersed thyroid preparation, so that any antibody present may have an opportunity for fixation. This substrate with its attached antibody is then thoroughly and repeatedly washed, so that adherent non-antibody globulins are removed. It is then exposed to a Coombs serum (anti-gamma-globulin) of known titre. Any antibody on the substrate will combine with some of the Coombs serum, reducing the titre found on re-estimation. If no antibody has become fixed upon the substrate then no such reduction should take place.

Method

A finely divided thyroid substrate was prepared by cutting up a block of fresh tissue, freed as far as possible from excess fat and connective tissue, at 30–40 μ on a freezing microtome, sections being received into saline. Thorough disintegration was achieved in a blender, the suspension washed twice in saline at 5° C., and then stored, until required, at −20° C.

Amounts of the wet substrate, each of 15.0± 0.2 mg., were weighed out into each of a batch of six Wassermann tubes. To each tube 5 drops of serum (from a standard-sized pipette) were added. In earlier experiments two control sera were incorporated in each batch of six tubes. Recently, as confidence in the method has grown, only one has been used. Control sera were obtained from blood transfusion donors. Sera plus substrates were incubated for 30 minutes at 38° C. in a water-bath and the substrate subsequently washed eight times with saline at room temperature. Coombs serum (Burroughs Wellcome) was diluted 1 in 64 and 10 drops (from a standard pipette) added to each of the substrates. These were now incubated for 20 to 30 minutes at 38° C. and the Coombs serum then removed by centrifugation. From each sample of Coombs serum dilutions down to 1:2,048 were made, and these were plated out on a glass plate. To each drop, 1 drop of sensitized Group O Rh+ erythrocytes (approximately in 10% suspension) was added and agglutination phenomena recorded at five and again at 10 minutes.

Results

The method was subjected to rigorous test by the assay of 100 "blind" sera kindly supplied by Professor G. A. Smart and Dr. S. G. Owen. The results showed very good correlation with the assessments previously made by Dr. Owen using the tanned red-cell method. Cases of Hashimoto's

disease were diagnosed clinically (Professor Smart); some were confirmed histologically.

To the 54 "blind" controls in the series, 20 known blood-donor controls were added. Four gave misleading results and two of these turned out negative on repetition. Of the other two, one was weakly and the other strongly positive. The latter was from a case of disseminated lupus erythematosus.

Positive results were obtained in 16 of 18 cases of Hashimoto's disease in which the presence of circulating antibody had been demonstrated by a positive tanned red-cell test. The two negative sera were from histologically confirmed cases of Hashimoto's disease in which the tanned red-cell test yielded slight and moderate antibody concentrations respectively.

Positive results with an anti-gamma-globulin consumption test were also obtained in 10 of 15 cases of Hashimoto's disease in which the tanned red-cell test had been negative. In these 10 cases, the test was strongly positive in four, moderately strong in four, and weak in three.

The anti-gamma-globulin consumption test was also positive in all of seven cases of myxoedema with positive tanned red-cell tests.

Discussion

From these results it can be seen that the anti-gamma-globulin consumption test is a valuable adjunct to the serological diagnosis of Hashimoto's disease. Whilst it is positive in the great majority of cases which give a positive tanned red-cell test, it also picks out a considerable number of undoubted Hashimoto cases which yield negative results by this method.

This latter feature is of interest since Doniach, Hudson, and Roitt (1960) have recorded a case of classical Hashimoto's disease (Case 3) in which tanned red-cell, complement-fixation, and precipitin tests were all negative; the serum of this case, nevertheless, contained antibodies demonstrable by Coons' fluorescent antibody technique. In such cases the anti-gamma-globulin test might be informative.

The exact nature of the substrate in the test as described is not clear. Since it is washed so many times the greater part of its thyroglobulin may be presumed lost, and it appears to consist principally of the fibrous stroma of the gland, with a certain amount of parenchymatous debris. This raises the question as to whether yet another antigen may be present in the whole gland.

The authors would like to thank Professor G. A. Smart and Dr. S. G. Owen, who kindly provided all the "blind" sera and co-operated in checking the results against the "key" in their possession; Dr. S. M. C. Murray and Dr. T. Davies, of the Regional Blood Transfusion Centre, for provision of control blood specimens; Mr. R. A. Brack for technical advice; Miss C. Smith for technical assistance; and the Medical Research Council for financial support.

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J. clin. Path. (1960), 13, 450.

STUDIES ON REANIMATION WITH PLASMA EXPANDERS

BY

C. T. NICOLAU, CECIL POPPA, M. IALOMITEANU, R. PÎRVU, AND M. IONESCU

From the Institute of Haematology, Bucharest, Rumania

(RECEIVED FOR PUBLICATION APRIL 16, 1960)

In dogs very severe haemorrhage, which leads to a state barely distinguishable from death, can be cured by one intra-arterial injection of dextran equal in volume to three-quarters or more of the estimated blood loss, provided that the dextran injection contains adrenaline and lobeline. Recovery after this treatment is only temporary, and a blood transfusion of about 25% of th e subject's lost volume of blood is needed for complete recovery.

It may be possible to apply these results to man, in which case, the reanimation method of Negovsky could be simplified and therefore be easy to use in any circumstances.

The progress of surgery during the last 20 years has depended largely on the improvements in the techniques of anaesthesia, the discovery and application of antibiotics, and the widespread use of blood transfusion. During the last 10 years the use of blood transfusion has attained first importance in treating patients suffering from surgical shock and particularly those who have bled freely. In Britain the infusion of blood, plasma or plasma substitutes, usually intravenously, sometimes by the intra-arterial route (Devitt and Widgerow, 1951; Bingham, 1952; Brown, 1953; Horton, Inkster, Mackenzie, and Pask, 1953; Maloney, Smythe, Gilmore, and Handford, 1953), has been the treatment of choice in all patients who have lost large volumes of blood, but Negovsky in the Soviet Union has developed a more complex method applicable even to patients on the brink of death. He injects intra-arterially blood to which adrenaline has been added and at the same time introduces an intratracheal tube through which oxygen is taken to the lungs (Negovsky, 1945 and 1954).

We have previously shown (Nicolau and Poppa, 1958) that this method will prevent clinical death even when a macromolecular substance, such as dextran, is used in place of blood. Recovery from shock achieved in this way is temporary, lasting only six to eight hours, and for permanent recovery blood must be administered subsequently.

For the success of the Negovsky method intratracheal insufflation of oxygen is essential, but this is inconvenient. In this paper we report our attempts to relieve post-haemorrhagic shock by perfusion of dextran and adrenaline with the addition of lobeline. The hypothesis was that the direct action of lobeline on the respiratory centre should make oxygen therapy unnecessary.

Experimental

Studies were performed on 26 dogs (weight 14–16 kg.), of which eight were controls which received saline in place of dextran, adrenaline, and lobeline.

Procedure.—Blood volume was determined with T 1824 (Porat, 1951) and narcosis with sodium evipan (30 mg./kg. body weight). Heparin was given in a dose of 4 mg. (250 I.U.)/kg. body weight. Blood was then removed from the carotid artery at the rate of 150 ml. per minute until 60 to 85% of the total blood volume had been removed. Dextran was perfused into the carotid artery (centripetal) at a rate of 150 ml. per minute and a pressure of 180 to 200 mm. of mercury, and 1 mg. adrenaline and 3 mg. lobeline injected with the blood substitute.

Blood pressure records were obtained with a mercury manometer connected with the femoral artery, and the respiratory movements from a pneumograph used with a Marey capsule. Records were made on smoked paper using Jaquet's electrokymograph with 6-second markers.

The experimental animals, after bleeding, were divided into two batches.

Group 1.—Nine animals received dextran equal in volume to the blood removed, and thereafter a blood transfusion equal to one-quarter of the blood removed; in three animals at four hours after dextran, in three at six and three at eight hours.

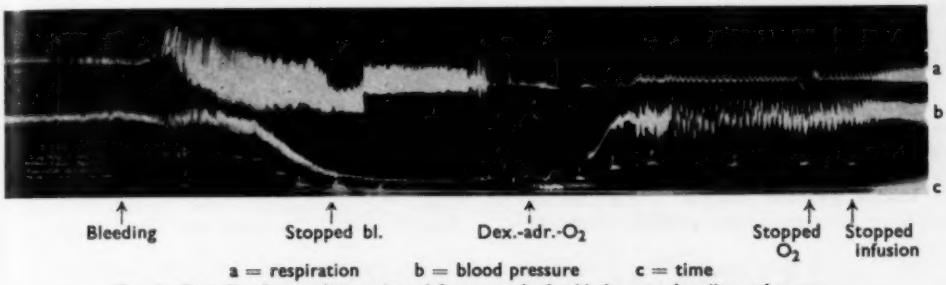

FIG. 1.—Recording from a dog reanimated from near death with dextran, adrenaline, and oxygen.

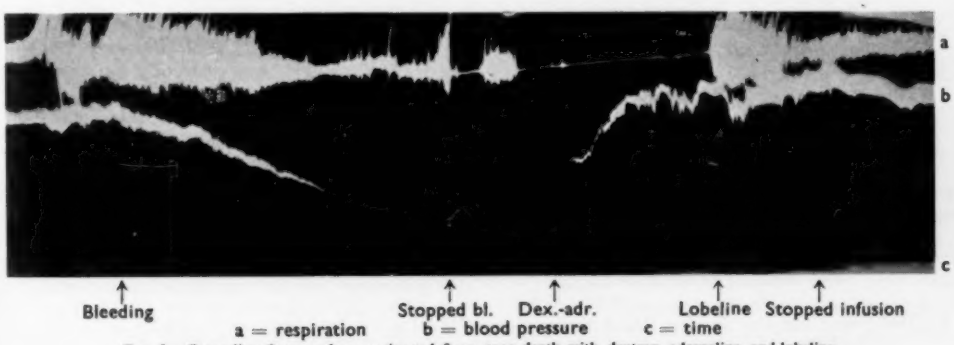

FIG. 2.—Recording from a dog reanimated from near death with dextran, adrenaline, and lobeline.

Group 2.—Nine animals also received dextran equal to 75% of the blood removed and thereafter a blood transfusion of 25% of the blood removed; in three animals at three hours, in three at five hours, and in three at seven hours after bleeding.

The controls received saline in place of dextran with adrenaline and lobeline, and thereafter a 25% blood transfusion was given at four and six hours.

Results

All the animals in the first two batches recovered completely. Of the control animals, six died within two hours and two recovered.

Discussion

One of the important points of the Negovsky method of resuscitation is the use of intratracheal insufflation. We have found that resuscitation depends on the simultaneous recovery of respiratory function (which seems to be achieved by intratracheal insufflation) and the vasomotor automatic reflex, which depends on stimulation of the pressure receptors in the arterial tree. Restoration of blood volume and even of circulation does not necessarily save the animal's life. Spontaneous respiratory activity must be established, and, with the original Negovsky method, this could be accomplished only by the intratracheal route. We doubted whether supply of large quantities of oxygen is really important, because exsanguinated animals have a very low metabolic rate, and we have previously demonstrated (Nicolau and Poppa, 1958) that the small volume of blood remaining in the vessels, the liver, and the spleen is enough to carry oxygen sufficient to keep the tissues alive. We therefore considered that the effect of oxygen treatment was to stimulate by a reflex route the respiratory centre in the bulb, which could be effected directly by some other drug, for example lobeline, which has been used for this purpose in newborn children for many years. This hypothesis was confirmed by the experiments reported here.

Conclusion

We consider that this may have great importance in medical practice, in small hospitals, maternity homes, or in emergencies where it is difficult to establish recovery from severe haemorrhagic shock, because the simultaneous performance of intra-arterial transfusion and of tracheal intuba-

tion is rarely possible. Since we have shown that tracheal intubation is unnecessary it should now be possible to treat post-haemorrhagic shock by the intra-arterial administration of plasma expanders (in this instance dextran) combined with adrenaline and lobeline, provided that a blood transfusion of about one-quarter of the blood loss is given within six to eight hours of the initiation of treatment. The blood provides the vehicle for the oxygen which cannot be carried by macromolecular plasma expanders.

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Association of Clinical Pathologists Broadsheets

The following broadsheets (new series) are published by the Association of Clinical Pathologists. They may be obtained from **Dr. F. Hampson, Department of Pathology, Royal Berkshire Hospital, Reading**, price 1s. each.

No.	Title	Date	Author
1	The Disc Technique for the Rapid Determination of Bacterial Sensitivity to Antibiotics	1952	R. W. Fairbrother
2	Determination of Sensitivity of *M. tuberculosis* to Streptomycin	1952	R. L. Vollum
3	The Detection of Barbiturates in Blood, Cerebrospinal Fluid, Urine, and Stomach Contents	1953	L. C. Nickolls
4	The Estimation of Carbon Monoxide in Blood	1953	D. A. Stanley
5	The Identification of Reducing Substances in Urine by Partition Chromatography on Paper	1953	G. B. Manning
6	The Paul-Bunnell Test	1954	R. H. A. Swain
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13	The Identification of Serotypes of *Escherichia coli* Associated with Infantile Gastro-enteritis	1956	Joan Taylor
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15	The Estimation of Faecal "Urobilinogen"	1957	C. H. Gray
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19	The Laboratory Diagnosis of Fibrinogen Deficiency	1958	R. M. Hardisty
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22	Investigation of Haemolytic Anaemia	1959	J. G. Selwyn
23	The Dried Disc Technique for Bacterial Sensitivity Tests	1959	R. W. Fairbrother and J. C. Sherris

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THE LATEX SLIDE TEST IN RHEUMATIC DISORDERS

BY

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From the Royal Bath Hospital, Harrogate

(RECEIVED FOR PUBLICATION JULY 11, 1960)

The latex slide test (L.S.T.) has been shown to be simple to perform and easy to read. It gives results comparable with the more complicated serological procedures used in the diagnosis of rheumatoid arthritis.

In 603 cases the L.S.T. and differential agglutination test gave a high degree of correlation. There were only four discrepancies between the two tests. The L.S.T. is the more practicable for routine laboratory use.

The L.S.T. has been correlated with the duration, activity, and extent of rheumatoid arthritis, and also with the erythrocyte sedimentation rate, haemoglobin level, and radiographic appearances. The various serological tests are tabulated.

It has repeatedly been shown that the serum of a large percentage of patients with rheumatoid arthritis gives a characteristic agglutination reaction often to a high titre. Many techniques have been employed to demonstrate this reaction. We have evaluated clinically and serologically the latex slide test (L.S.T.) and compared it with the differential agglutination test (D.A.T.).

Methods and Material

The L.S.T. was carried out according to the method described by the makers (Hyland Laboratories). In principle this involves the use of polystyrene latex particles coated with a globulin-containing reagent, and their subsequent flocculation by rheumatoid serum. The test was read macroscopically after one minute and the result designated positive, weak positive, or negative depending on the degree of flocculation.

The D.A.T. was carried out by a modification of the techniques of Rose, Ragan, Pearce, and Lipman (1948) and Heller, Jacobson, Kolodny, and Kammerer (1954), described by Greenbury (1957). A differential titre of 16 or above was considered positive. Haemoglobin was measured by the alkaline haematin method (100% = 14.8 g. haemoglobin per 100 ml. blood), and the erythrocyte sedimentation rate by the Westergren method.

Six hundred and three patients examined at the Royal Bath Hospital between November, 1958, and March, 1960, were assessed, the diagnosis being made without reference to the serological results. Patients were also classified according to the criteria laid down by the American Rheumatism Association (Ropes, Bennett, Cobb, Jacox, and Jessar, 1959), using the categories definite, probable, and possible. The extent of the disease was graded as severe, moderate, or mild; the activity as severe, moderate, or inactive. Radiographs were taken where indicated.

Serological Comparison

Of the 603 patients, 232 had a positive L.S.T., 24 had a weakly positive L.S.T., and 347 gave a negative result. In only four instances was there a discrepancy between the two tests and in each of these cases the L.S.T. was positive while the D.A.T. was negative. A close correlation was found between the D.A.T. titre and the L.S.T. results. All sera giving a weak positive L.S.T. produced a D.A.T. of 64 or less, with one exception. This was from a man aged 58 years, with osteoarthritis for seven years, who had a D.A.T. of 1,024 and a weakly positive L.S.T.

The four patients whose tests were not in agreement are shown in Table I. The two with definite rheumatoid arthritis were women in the third decade with histories of four and six years respectively. The third patient was a woman aged 68 years with primary generalized osteoarthritis of 11 years' duration who had some features suggesting rheumatoid arthritis. The fourth patient was a 62-year-old man with a history of generalized osteoarthritis for 18 years.

General Survey of Results

The results of the L.S.T. in relation to the diagnoses are shown in Table I. The L.S.T. was positive in 85% of patients with definite or probable rheumatoid arthritis, but less than half the cases in the "possible" group were positive.

TABLE I
RESULTS OF D.A.T. AND L.S.T. IN RHEUMATIC DISORDERS

Clinical Diagnosis	Total	D.A.T. and L.S.T. Positive	D.A.T. Negative L.S.T. Positive	Percentage L.S.T. Positive
Rheumatoid arthritis (definite and probable)	246	206	2	85
Rheumatoid arthritis (possible)	48	20	1	44
Osteoarthritis	131	5	1	5·3
Psychogenic or muscular rheumatism	53	1	—	2
Lumbar disc lesion	25	0	—	—
Cervical spondylosis	18	1	—	—
Ankylosing spondylitis	12	1	—	—
Psoriasis and arthritis	14	4	—	—
Still's disease	11	1	—	—
Systemic lupus erythematosus	6	4	—	—
Gout	6	2	—	—
Caplan's syndrome	3	3	—	—
Miscellaneous	30	4	—	—
	603	252	4	

It was found that only nine out of 233 patients in whom there was no suggestion of rheumatoid arthritis had a positive test (3.8% "false positives"). The corresponding figure for 131 patients with osteoarthritis was 5.3%, emphasizing the usefulness of the test in differentiating these cases from rheumatoid arthritis. The absence of a positive L.S.T. in all but one of the patients with muscular rheumatism indicates the value of the test in excluding rheumatoid disease.

Patients with psoriasis and atrophic arthritis have been tabulated separately since there are good reasons for considering that they contain a group with an arthritis distinct from rheumatoid arthritis (Wright, 1959). It was interesting to note that only one patient with ankylosing spondylitis and one case of Still's disease had positive tests.

The miscellaneous group comprised eight patients with periarthritis of the shoulder (one positive), four with tenosynovitis, two with rheumatic fever, three with Reiter's syndrome, two with palindromic rheumatism (one positive), and two with Raynaud's disease (one positive). There were single cases of subacute bacterial endocarditis, undulant fever, dermatomyositis, colitic arthritis, pyogenic arthritis, spinal tumour, monarticular arthritis, and traumatic arthritis which were all negative, and finally a case of intermittent hydrarthrosis which was positive.

Findings in Rheumatoid Arthritis

Using the diagnostic criteria laid down by the American Rheumatism Association, patients with rheumatoid arthritis were classified as definite, probable, and possible. Of 216 in the first group 90% had a positive test; the probable group comprised 58 patients of whom 55% were positive, whilst in the remaining group of 20 with possible rheumatoid arthritis only four (20%) had a positive L.S.T. In all groups, the males showed a consistently higher percentage of positive results.

The results of the L.S.T. in relation to duration of disease in the definite and probable rheumatoid arthritics are shown in Fig. 1. The lower percentages of positive tests occurred in the early and long-standing groups. However, of 16 patients seen within six months of onset, 12 tests were positive, and a further one was weakly positive.

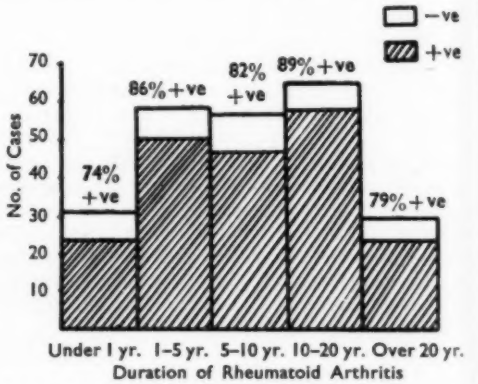

FIG. 1.—Results of the L.S.T. related to the duration of rheumatoid arthritis.

Patients with arthritis which was mild in extent showed a smaller percentage of positive results (74%) than those with more severe disease (89%). Thirty-one patients had subcutaneous nodules, i.e., 18% of those with definite rheumatoid arthritis, and in every case the L.S.T. was positive.

When activity of disease was graded on clinical grounds only, those with inactive rheumatoid arthritis yielded 54% positive results compared with 88% in those with active disease. The administration of steroids did not alter the incidence of positive tests. No correlation was found between the L.S.T. and activity of disease as measured either by erythrocyte sedimentation rate or haemoglobin levels.

One hundred patients had radiographic bone changes typical of rheumatoid arthritis. Of the 30 men in this group all but one had positive tests, and the corresponding figure for the 70 women was 70%. The overall figure was 85% positive in the presence of typical radiographs.

Discussion

A large number of tests are now available for the demonstration of rheumatoid factor in the blood of patients with rheumatoid arthritis. Most of these use an indicator which may be biologically active or inactive as classified in Table II.

The majority of these techniques are tedious and time consuming. Recently a slide test (the L.S.T.), which is both easy to perform and gives rapid results, has been developed commercially. In over 600 cases we have demonstrated that it is a reliable test in rheumatic disorders. The parallel study of the D.A.T. showed only four occasions on which the tests were not in agreement.

TABLE II

I. Indicator Biologically Active
  1. *Sheep Cells*
  (*a*) *Agglutination*. The differential sheep cell agglutination test, described by Rose *et al.* (1948) (D.A.T. or S.C.A.T.)
  This classical test may be modified in several ways:
    (1) Heterophil antibodies absorbed (Heller *et al.*, 1949)
    (2) Sheep serum used as the diluent (Heller *et al.*, 1952)
    (3) Sheep cells treated with tannic acid and coated with human globulin (Heller *et al.*, 1954)
    (4) Rheumatoid factor isolated as euglobulin by dialysis (Ziff *et al.*, 1956).
  (*b*) *Inhibition Test* (Ziff *et al.*, 1956)
  2. *Human Erythrocytes*
  Sensitized human D erythrocyte test (Waller and Vaughan, 1956)

II. Indicator Biologically Inactive
  1. *Latex Particles*
  (*a*) *Agglutination*. Standard latex fixation test (Singer and Plotz, 1956)
  This may be modified as follows:
    (1) "Drop" and tube methods, L.F.T. (Rheins *et al.*, 1957; Thomas *et al.*, 1957)
    (2) Latex slide test (L.S.T.)
    (3) Using euglobulin
  (*b*) *Inhibition Test* (Hall *et al.*, 1958)
  2. *Bentonite*
  Flocculation test (B.F.T.) (Bozicevich *et al.*, 1958)
  Slide test

III. No Indicator Used
  F11 precipitation test (Epstein *et al.*, 1956)

There is evidence that sera containing greatly elevated gamma globulins, e.g., syphilis and disorders of the liver, may produce more false positive results with the L.S.T. than with the D.A.T., but these conditions rarely give rise to confusion in the differential diagnosis of rheumatic disorders. For routine laboratory use, therefore, the L.S.T. would appear to be the most practicable of the tests at present available in this field. For research purposes the quantitative aspects of the D.A.T. may be advantageous, although it should be noted that the L.S.T. provides a roughly quantitative measure, and correlates well with the titre of the D.A.T.

Patients with rheumatoid arthritis were classified without knowledge of the serological results to ensure an unbiased study. Of 246 patients with definite or probable rheumatoid arthritis, 85% gave a positive result. On the criteria laid down by the American Rheumatism Association 43 more were classified as definite than by our clinical criteria. The weakness of the American Rheumatism Association classification is the equal weight given to each item. For instance, nodules score no more than a single swollen joint, although by many they are regarded as pathognomonic of rheumatoid arthritis, and in our series all cases with nodules had a positive L.S.T.

Extent of disease may have some bearing on the L.S.T. since those with mild arthritis had fewer positive results. To test the hypothesis that burnt-out rheumatoid arthritis gives a negative test, the L.S.T. was related to the activity of the arthritis. Inactive disease judged clinically was found to show fewer positive results. On the other hand, activity judged by the E.S.R. and haemoglobin did not correlate with the L.S.T. and gave no support to the hypothesis. The presence of radiological changes of rheumatoid arthritis increased the likelihood of the test being positive, but 15% of patients with such changes had a negative result. In keeping with the results of other workers using the D.A.T. (Ball, 1952), the incidence of false positive tests was low (3.8%).

We are indebted to Professor S. J. Hartfall for his encouragement in this study, and to Mr. S. Wisdom, chief technician, for technical assistance and willing co-operation.

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J. clin. Path. (1960), 13, 456.

BOOK REVIEWS

Tools of Biological Research. Edited by Hedley J. B. Atkins, with an Introduction by Sir Cyril Hinshelwood. (Pp. xvi+183; 113 figures. 37s. 6d.) Oxford: Blackwell; Toronto: Ryerson Press. 1960.

This volume deals with a number of modern research techniques and will therefore be welcomed by a wide variety of specialists in medicine. In his preface the editor explains how, at the meetings of the Surgical Research Society, some senior members found themselves unfamiliar with many of the tools being used in research projects under discussion. Accordingly a two-day symposium was organized at Guy's Hospital, London, on 10 selected "tools of biological research."

On reading this book one becomes more and more impressed by the amount and variety of useful information which it contains. The research worker contemplating a new technique will find the appropriate chapter quite invaluable. In addition to describing the actual technical procedure, its uses and applications are clearly discussed and a very ample selection of references given for further reading.

The introductory address by Sir Cyril Hinshelwood is followed by a most stimulating chapter by Dr. Armitage on the use of statistics in the design of experiments. Fundamental techniques such as "Electron Microscopy," by Dr. Robertson, and "Phase Contrast and Interference Microscopy," by Mr. Barer, are discussed in a way that is most attractive and comprehensible to those with a non-technical training. Clinical pathologists will be interested to read the chapters by Prof. Thompson and Dr. Baker on flame photometry and by Prof. Martin on electrophoresis. Prof. Martin is to be congratulated on his insistence that the basic design of electrophoresis apparatus is of the first importance if reliable results are to be obtained. Pathologists and many others will be fascinated by the excellent chapter on tissue culture by Dr. Honor Fell. As the leading exponent of the subject, and indeed as one of the pioneers in this country, she writes with great clarity in defining the various types of tissue culture, their aims, and their applications. Appropriately, this chapter is followed by a chapter on tissue transplantation by Dr. Brent, who outlines the present achievements and the barriers which at the moment prevent further progress in this technique which may well play a large part in the future of surgery. Medical specialists will be pleased to see a chapter on electromanometry by Dr. Shillingford and one on mass spectrometry by Dr. Hugh Jones. These applications of electronics to the techniques of cardiac catheterization and blood gas analysis have done much to contribute to the enormous advances made in recent years in diseases of the heart and lungs. Radiology has not been neglected, and future improvements are foreshadowed in a lucid account of image intensification by Drs. Combee and Botden.

The book itself is admirably produced, containing diagrams which are clear and illustrations which are of a high quality. The full title of the papers given in the lists of references is a practice which might well be followed by other publishers. It is certain that this work will be widely appreciated, and Mr. Hedley Atkins is greatly to be congratulated on the happy inspiration which resulted in the holding and publication of this symposium. GEORGE CUNNINGHAM.

Diagnostic Radioisotopes. By Charles A. Owen, Jr. (Pp. xix+425; 70 figures, 49 tables. £6 6s.) Oxford: Blackwell. 1959.

A number of competent books on this and related subjects have been published in recent years, and it is against these that this book must be judged. All of them face a common problem: that of achieving a satisfactory balance between, on the one hand, discussion of the purely physical problems of radioactivity measurement, and the theoretical considerations upon which the isotopic techniques are based, and, on the other hand, descriptions of the methods currently in use and interpretation of results. Some succeed better than others in the balance achieved; this book fails completely.

The chapters on counting techniques are largely useless and are in no way improved by the many illustrations of boxes of electronic equipment which have no difficulty in preserving their anonymity behind their front panels. The theoretical bases of some of the problems are poorly explained, others, e.g., those involving multicompartment systems, are not discussed.

The allotment of space to methods and interpretations bears little relation to their importance: for instance, 10 pages are devoted to the problem of B_{12} absorption, but only 13 to the whole of the cardiovascular system, electrolyte and fluid spaces, and protein metabolism. Indeed, these latter subjects cannot be said to have been properly presented at all.

There are extensive but largely uncritical reviews of the literature relating to medical isotopes, and 64 out of the 381 pages of text are devoted to pointless tables including logarithm tables and that ubiquitous du Bois surface area nomogram. C. J. HAYTER.

Eight Colloquia on Clinical Pathology

We regret that in the review of the "Eight Colloquia on Clinical Pathology" (*J. clin. Path.*, 13, 278) we criticized the apparent delay in publication. The review copy was sent direct to the Editor of this journal by the publishers at the request of the Editors of the Colloquia, but they did not point out that the Colloquia had in fact been distributed to the participants within a year of the end of the Congress. We apologize to Professor Welsch and his colleagues for our misunderstanding.—ED.

J. clin. Path. (1960), 13, 457.

THE ASSAY AND PROPERTIES OF LABILE FACTOR (FACTOR V)

BY

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Human oxalated plasma stored at 4° C. until the prothrombin time is increased beyond 60 sec. is a reliable medium for assaying labile factor (factor V) because its response to added labile factor corresponds quantitatively to that of plasma from patients with congenital deficiency of this factor. Such an agreement is not obtained with plasma stored at 37° C. The stability of labile factor is closely associated with ionized calcium. The addition of thrombin to fresh oxalated plasma causes an apparent hyperactivity of labile factor, but this is completely removed by adsorption with $Ca_3(PO)_2$. Oxalated plasma when adsorbed with $Ca_3(PO_4)_2$ before treatment with thrombin does not develop this adventitious activity, nor does it occur in stored plasma treated with thrombin. The seemingly high labile factor activity in serum can be explained by the activation of this factor which is independent of labile factor but acts synergistically with it. The true labile factor concentration can be determined only after the accelerator is removed by adsorption with $Ca_3(PO_4)_2$. A close agreement between the consumption of prothrombin and the loss of labile factor during clotting is observed.

The discovery in 1943 of a new clotting factor now commonly known as labile factor or factor V, which was not recognized in the classical theory of blood coagulation formulated by Morawitz in 1904, initiated a radically new approach to the study of coagulation mechanisms and the haemorrhagic diseases. Owren (1947a) described the first case in which a bleeding state was caused by lack of a factor which appeared to be similar or identical with the one which is decreased when oxalated human plasma is stored at 4° C. On the assumption that the prolonged prothrombin time of such stored plasma is caused specifically by a loss of labile factor, a method of assay using a stored human plasma as the medium was developed by Quick and Stefanini (1948). With this procedure, it was shown that human blood contains a much lower concentration of labile factor than the blood of the common laboratory animals. Since there had been no opportunity to compare stored plasma directly with that from a patient with congenital deficiency of labile factor, the unequivocal reliability of the assay method could not be established. With the availability recently of blood from three siblings having strikingly low concentrations of labile factor, such a comparative study could be made, and it prompted a reinvestigation of this clotting factor.

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Material and Methods

Collection of Blood.—A silicone-coated needle and syringe were used and the blood was transferred to test-tubes similarly coated. These were kept in an ice bath until used.

One-stage Prothrombin Time.—The exact procedure as previously described (Quick, 1957) was followed. This included using sodium oxalate as the anticoagulant, acetone-dehydrated rabbit brain as the thromboplastin reagent, and 0.01M $CaCl_2$ for recalcification. A prothrombin time of 12 sec. for fresh normal human plasma was mandatory in the control for each experiment.

Adsorption of Plasma to Remove Prothrombin and Stable Factor (Factor VII).—One millilitre of a suspension of 0.012M $Ca_3(PO_4)_2$ contains the correct amount of adsorbent to remove completely the prothrombin and stable factor (factor VII) from 1 ml. of oxalated rabbit plasma. The required volume of the suspension is pipetted into a test-tube and the $Ca_3(PO_4)_2$ is packed by centrifugation. The supernatant water is poured off, and after the tube is drained the plasma is added and mixed with the $Ca_3(PO_4)_2$. After five minutes the adsorbent is removed by high centrifugation.

Results

Comparison of Stored Human Plasma with Plasma from Subjects Having Congenital Deficiency of Labile Factor.—On adding an equal volume of fresh normal oxalated plasma either to plasma from a patient with congenital deficiency of labile factor or to stored plasma, a mixture is obtained which has approximately a normal prothrombin time (11½–13 sec.) (Table I). No correction is obtained when labile-factor-deficient plasma is mixed with stored plasma. On adding fixed amounts of deprothrombinized rabbit plasma, which has a high concentration of labile factor, to the patient's plasma and to stored plasma, the correction of the prothrombin time is quantitatively the same in both types of plasma except that, with increasing amounts, a minimum prothrombin time of 8 sec. is obtained in stored plasma and 11½ sec. in the patient's fresh plasma. When the patient's plasma is stored for 48 hours in glass at 4° C., a minimum prothrombin time of 8½ sec. is then obtained. A probable explanation for this will be offered in the discussion.

TABLE I

COMPARISON OF PLASMA FROM PATIENTS HAVING CONGENITAL LABILE FACTOR DEFICIENCY WITH STORED PLASMA IN THE RESPONSE TO ADDED LABILE FACTOR

	Prothrombin Time (sec.)		
	Patient 1*	Patient 2	Stored Plasma
Undiluted plasma	53	42	95
Plasma diluted with an equal volume of normal plasma	12	13	11·5
Plasma diluted with an equal volume of stored plasma	55	48	95
Amount of deprothrombinized rabbit plasma added to 0·1 ml. plasma:			
0·001 ml.	23	20	23
0·005	14	15	14
0·01	12	11·5	11
0·02	12	12	10
0·05	11·5	11·5	8
0·05	8·5†	8·5†	

* The basic prothrombin consumption time of Patient 1 was 10·5 sec. When 0·1 ml. of deprothrombinized rabbit plasma was added to 1 ml. of the patient's blood before clotting, the prothrombin consumption time was 45 sec.
† After 48 hours' storage in glass.

The results in Table I show that normal plasma stored at 4° C. appears to be nearly identical to plasma from patients with congenital labile factor deficiency. The ease with which labile factor can be diminished by storage at 4° C. makes the preparation of plasma suitable for assaying labile factor very simple.

The method of Wolf (1953) for preparing plasma for assaying labile factor was found unsatisfactory. He incubates oxalated plasma at 37° C. for 24 hours. From the results presented in Table II, it is evident that changes other than loss of labile factor occur in the plasma, so that the addition of excess labile factor does not bring the prothrombin time to normal as it will when added to plasma stored at 4° C.

TABLE II

COMPARISON OF HUMAN OXALATED PLASMAS STORED AT 4° C. FOR 14 DAYS AND AT 37° C. FOR 24 HOURS IN RESPONSE TO ADDED LABILE FACTOR

	Prothrombin Time (sec.)		
	Fresh	Stored at 4° C. for 14 Days	Stored at 37° C. for 24 Hours
Deprothrombinized rabbit plasma added to 0·1 ml. of plasma:			
0 ml.	12	60	240
0·01 ,,	12	10	50
0·05 ,,	11	8	18

Assay Method for Labile Factor.—Normal oxalated plasma is distributed in 5 ml. portions to test-tubes which are placed in a refrigerator kept at 4° C. The prothrombin time is determined after seven days, and again at several days' intervals, until it has increased to 60 sec. This requires about a fortnight. The plasmas are pooled and stored at −20° C.

The labile factor curve is based on the finding that when 1 vol. of fresh human plasma is mixed with 9 vol. of stored plasma, the mixture has a prothrombin time which averages about 18 sec. The usual range is 17 to 20 sec. A plasma giving a correction of 18 sec. is arbitrarily designated as having a labile factor concentration of 100, and, since 1 vol. of this plasma is mixed with 9 vol. of stored plasma which has a concentration of less than 1%, the 18 sec. can be regarded as the

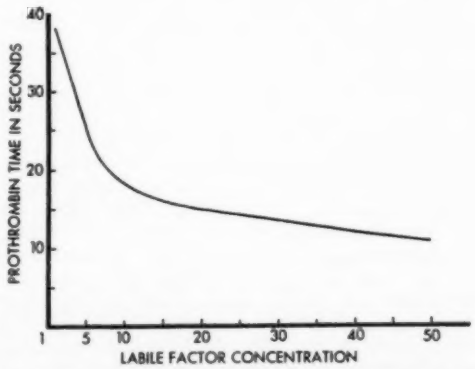

FIG. 1.—The relation of the prothrombin time to the concentration of labile factor (factor V) in human plasma. The concentration of labile factor was calculated from the dilution of fresh normal plasma with aged plasma.

prothrombin time of a plasma containing 10% of labile factor. By means of varying dilutions of stored plasma with fresh, values are obtained from which a curve can be constructed correlating the prothrombin time with labile factor deficiency (Fig. 1). It will be noted that the prothrombin time becomes 12 sec. when 4 vol. of fresh plasma is mixed with 6 vol. of stored plasma. Fresh plasma contains over twice as much labile factor as is required to maintain the prothrombin time at 12 sec.; consequently, over half can be lost before the prothrombin time is prolonged.

In carrying out the test, the plasma to be assayed is adsorbed with $Ca_3(PO_4)_2$ at 4° C. This removes very little labile factor, but it does take out an accelerator which may be present. One-hundedth of a millilitre of the adsorbed plasma is mixed with 0.09 ml. of stored human plasma and the prothrombin time of the mixture determined. The value in terms of percentage of labile factor is read from the chart and multiplied by 10 because of the dilution.

The assay method is sensitive to small changes in labile factor even in the range before the prothrombin time is affected. This is illustrated by the simple experiment of incubating oxalated human plasma for 30 min. at 37° C. Owren (1947b) states that no deterioration of factor V occurs in this short period, yet when studied by our method (Table III) it is found that the activity decreased from 120% to 86%, which is a drop of 28%.

Concentration of Labile Factor in Various Laboratory Animals.—The determination of labile factor in the blood of the common laboratory animals is simple because the concentration is much higher than in human plasma. One need only determine how much the plasma has to be diluted to correct the prothrombin time of stored human plasma to 18 sec., which has been set as the normal correction when 1 vol. of fresh human plasma is mixed with 9 vol. of stored plasma. In determining the labile factor, the animal plasma is diluted with saline in various proportions. To 0.09 ml. of stored plasma, 0.01 ml. of the diluted plasma is added and the prothrombin time determined. From the prothrombin time obtained, the concentration of labile factor is calculated. The outstanding finding is the high concentration of labile factor in rabbit plasma and the low level in human plasma (Table IV).

TABLE III

EFFECT OF INCUBATING OXALATED PLASMA AT 37° C. ON LABILE FACTOR

	Prothrombin Time (sec.)		Labile Factor (%)
	Basic	Mixed with 9 Vol. of Aged Plasma	
Before incubation	12	17	120
After 30 min. incubation ..	12	19	86
,, 120 ,, ,, ..	17	30	29

TABLE IV

RELATIVE CONCENTRATION OF LABILE FACTOR (FACTOR V) IN PLASMA OF VARIOUS SPECIES

Man	Rabbit	Dog	Cat	Cow	Sheep
100	5,000	1,000	350–500	800	300–500

TABLE V

EFFECT OF VARIOUS ANTICOAGULANTS ON PROTHROMBIN TIME AND LABILE FACTOR DURING STORAGE AT 4° C.

Anticoagulant	Time of Storage (hr.)	Prothrombin Time					
		In Glass			In Silicone		
		Basic (sec.)	Corrected for Labile Factor* (sec.)	Mixed with 9 Vol. Aged Plasma† (sec.)	Basic (sec.)	Corrected for Labile Factor* (sec.)	Mixed with 9 Vol. Aged Plasma† (sec.)
Sodium oxalate 0·1M ..	0	12	12	17	12	12	17
	24	14	8·5	29	16·5	12	26
	48	20	8	41	23	12	36
,, citrate 0·1M ..	0	12	12	17	12	11	17
	24	9	8	18	12	12	18
	48	10·5	8·5	20	13	11	17·5
,, ,, 0·2M ..	0	12	12	17	12	12	17·5
	24	13	8·5	21	14·5	12	20·5
	48	16	9	25	17·5	12	24
None	0				12	11	17
	24				12	11	16·5
	48				12	11·5	17

* To 0·09 ml. of the plasma, 0·01 ml. of deprothrombinized rabbit plasma was added. † To 0·09 ml. of aged plasma, 0·01 ml. of the plasma studied was added.

Effect of Anticoagulants on Labile Factor in Plasma Stored at 4° C.—Three types of plasma were studied: oxalated, citrated, and native. The results are recorded in Table V. The decrease of labile factor is greatest in oxalated plasma, but it is also fairly rapid when plasma is mixed with 0.2M sodium citrate. With 0.1M sodium citrate, the loss of labile factor is greatly retarded and in native plasma is almost negligible in the first 48 hours. The decrease of labile factor is not significantly different in glass or silicone-coated containers, but the prothrombin activity as measured by the prothrombin time after adding an excess of labile factor is increased in glass but remains practically unchanged in silicone.

Concentration of Labile Factor in Serum.—When human blood is clotted in glass, the resulting serum appears to have a higher labile factor activity than does the corresponding oxalated plasma as shown in Table VI. When the serum is adsorbed with $Ca_3(PO_4)_2$, this hyperactivity is lost and the concentration of labile factor is found actually to be low, suggesting that much of this factor is consumed during clotting. If platelet-poor plasma is clotted, the resulting serum also shows the apparent hyperactivity, which, after adsorption, is lost, but the residual concentration of labile factor is higher than in the serum obtained from the clotting of whole blood or platelet-rich plasma.

TABLE VI

LABILE FACTOR ACTIVITY IN SERUM

Stored oxalated plasma (ml.)	0·07	0·08	0·09
Serum from normal blood (Serum A) (ml.)	0·03	0·02	0·01
Prothrombin time (sec.)	5·5	6·5	8·0
Stored oxalated plasma (ml.)	0·07	0·08	0·09
Serum A adsorbed with $Ca_3(PO_4)_2$ (ml.)	0·03	0·02	0·01
Prothrombin time (sec.)	20·0	23·0	29·0
Stored oxalated plasma (ml.)	0·07	0·08	0·09
Serum from platelet-poor plasma (Serum B) (ml.)	0·03	0·02	0·01
Prothrombin time (sec.)	5·2	6·5	8·0
Stored oxalated plasma (ml.)	0·07	0·08	0·09
Serum B adsorbed with $Ca_3(PO_4)_2$ (ml.)	0·03	0·02	0·01
Prothrombin time (sec.)	12·0	13·0	16·0
Stored oxalated plasma (ml.)	0·07	0·08	0·09
Serum from platelet-rich plasma (Serum C) (ml.)	0·03	0·02	0·01
Prothrombin time (sec.)	7·5	7·5	8·2
Stored oxalated plasma (ml.)	0·07	0·08	0·09
Serum C adsorbed with $Ca_3(PO_4)_2$ (ml.)	0·03	0·02	0·01
Prothrombin time (sec.)	18·0	21·0	25·0

Action of Thrombin on Labile Factor in Oxalated Plasma.—When thrombin is added to oxalated plasma, the resulting defibrinated product has, like serum, a distinct hyperactivity when tested on stored plasma (Table VII), but adsorption with $Ca_3(PO_4)_2$ removes this increased activity. When oxalated plasma is adsorbed with $Ca_3(PO_4)_2$ before the addition of thrombin, no adventitious activity develops: likewise, in stored oxalated plasma, this activity fails to develop on treatment with thrombin.

TABLE VII

EFFECT OF THROMBIN ON LABILE FACTOR ACTIVITY

Stored oxalated plasma (ml.)	0·07	0·08	0·09
Fresh oxalated plasma (Plasma A) (ml.)	0·03	0·02	0·01
Prothrombin time (sec.)	12·2	13·5	17·5
Stored oxalated plasma (ml.)	0·07	0·08	0·09
Plasma A treated with thrombin* (Plasma B) (ml.)	0·03	0·02	0·01
Prothrombin time (sec.)	9·0	10·0	11·0
Stored oxalated plasma (ml.)	0·07	0·08	0·09
Plasma B adsorbed with $Ca_3(PO_4)_2$ (ml.)	0·03	0·02	0·01
Prothrombin time (sec.)	15·0	19·0	22·0
Stored oxalated plasma (ml.)	0·07	0·08	0·09
Plasma A adsorbed with $Ca_3(PO_4)_2$ (Plasma C) (ml.)	0·03	0·02	0·01
Prothrombin time (sec.)	13·0	16·0	18·5
Stored oxalated plasma (ml.)	0·07	0·08	0·09
Plasma C treated with thrombin* (ml.)	0·03	0·02	0·01
Prothrombin time (sec.)	12·5	14·5	17·0
Stored oxalated plasma (ml.)	0·07	0·08	0·09
Stored plasma treated with thrombin* (ml.)	0·03	0·02	0·01
Prothrombin time (sec.)	48·0	47·5	44·0

* Five units of thrombin were added to 1 ml. of plasma. The defibrinated plasma was kept at 4° C. for one hour before testing.

Discussion

The observation of various investigators that in congenital deficiency of labile factor the other plasma constituents which influence the prothrombin time are normal suggests that the plasma from such patients should be ideal for assaying the factor. It has, however, the disadvantage of not being readily available. The finding that normal plasma stored at 4° C. can be reliably substituted makes the quantitative study of labile factor possible and easy for any clinical laboratory.

The concentration of labile factor in human blood is much lower than in the common laboratory animals. This observation, which was made in the original study (Quick, 1943), has been confirmed by Murphy and Seegers (1948) and by Didisheim, Hattori, and Lewis (1959). The suggestion of Murphy and Seegers that the low level in man may "offer at least a partial explanation for the reported discrepancy of prothrombin values obtained by the one-stage and two-stage methods" is not supported experimentally. The concentration of labile factor in normal human plasma is not the limiting factor that fixes the prothrombin time (Quick, 1958). Over one-half of the labile factor must be lost before the

prothrombin time becomes prolonged as is shown in this study and was earlier reported by Alexander and DeVries (1949).

Confusion still exists concerning the stability and alteration of activity of labile factor. It was originally observed (Quick, 1943) that native chicken and goose plasma showed no increase of prothrombin time kept seven days in a refrigerator. When these plasmas were oxalated, the prothrombin time became markedly prolonged on storage. This suggests that the stability of labile factor is decreased in the absence of ionized calcium. In the present study it is shown that native human plasma loses little labile factor on storage in 48 hours, whereas the oxalated plasma does. Significantly, when 9 vol. of plasma is mixed with 1 vol. of 0.1M sodium citrate, the resulting mixture exhibits no increase in the prothrombin time; it actually shows a decrease. If, instead, 0.2M citrate is used, the same increase in prothrombin time is observed as in oxalated plasma. The difference in stability of labile factor in citrated and oxalated plasma was observed by Fahey, Ware, and Seegers (1948), but they offered no explanation. Since 0.1M sodium citrate does not completely depress the ionized calcium, it seems justifiable to conclude that the greater stability of the labile factor in the weaker citrate plasma is due to ionized calcium which is not completely depressed (Hussey, Quick, Stefanini, Consolazio, and Sargent, 1950).

When blood citrated with 0.1M citrate is stored in glass at 4° C., the prothrombin time decreases from the initial 12 sec. to about 9 sec., whereas no change occurs when stored in silicone. It is further observed that both the oxalated and citrated plasmas stored in glass have prothrombin times of 8 to 9 sec. when excess labile factor is added, but the same plasmas kept in silicone-coated test-tubes retain a prothrombin time of approximately 12 sec. Alexander and DeVries also noted this enhanced prothrombin activity on storage in glass and remarked that it could not be explained. Shortly before this, Quick and Stefanini (1949) formulated the hypothesis that the prothrombin in human blood is only partly free and that a large fraction is in an inactive state. On storage in glass the inactive prothrombin becomes activated, whereupon the prothrombin time is shortened. In both oxalated and citrated plasma this activation readily occurs in glass but not in silicone. Since the rate at which labile factor decreases in oxalated and in 0.2M sodium citrated plasmas is much greater than the speed of activation of prothrombinogen, the prothrombin time steadily increases. In the plasma with 0.1M sodium citrate, the reverse is true because the rate with which labile factor disappears is much slower than that of the activation of inactive prothrombin.

During storage of adult human plasma in glass at 4° C., two changes are occurring simultaneously: labile factor is diminishing and, according to the author's hypothesis, inactive prothrombin is being converted to the active form. The latter reaction, which is measurable by the one-stage prothrombin time, is masked unless the first is retarded, as occurs when plasma is mixed with 0.1M sodium citrate. These changes occur in the absence of thrombin. When oxalated plasma is treated with thrombin and incubated for one hour to permit inactivation of the added thrombin, the resulting defibrinated plasma has, when tested by our assay method, shown a marked increase in what appears to be labile factor activity. When, however, the defibrinated plasma is treated with $Ca_3(PO_4)_2$, this hyperactivity is lost and the adsorbed plasma is found to have a slightly lower concentration of labile factor than it had before treatment with thrombin. This clearly suggests that thrombin brings about the generation of another factor which acts synergistically with labile factor to shorten the prothrombin time markedly. If oxalated plasma is adsorbed with $Ca_3(PO_4)_2$ before treatment with thrombin, no hyperactivity develops. Likewise, thrombin fails to produce this adventitious activity in stored plasma. This factor which is activated by thrombin appears to be distinct and independent of labile factor. It is adsorbed by $Ca_3(PO_4)_2$ and is absent in stored plasma. It is probably identical with the agent Alexander, DeVries, Goldstein, and Landwehr (1949) named S.P.C.A. (serum prothrombin conversion accelerator).

The activation of this new factor explains the apparently high labile factor activity in serum. When, however, the serum is adsorbed with $Ca_3(PO_4)_2$, it is found that the labile factor has actually diminished. The more complete the clotting as measured by the prothrombin consumption test, the greater the decrease of labile factor, and conversely in defective clotting, such as occurs in haemophilia and thrombocytopenia, much labile factor remains in the serum. This was reported by Alexander, Goldstein, and Landwehr (1950) and again recently by Douglas (1956).

These observations suggest that labile factor enters the reaction as a reactant and not as an accelerator. At the time Fahey *et al.* (1948) carried out their studies on the factor which they called Ac-globulin, the S.P.C.A. of Alexander had not been recognized. The observation of greater

activity in serum was regarded as an activation of Ac-globulin; they distinguished between plasma Ac-globulin and serum Ac-globulin and postulated that the factor existed in plasma in an inactive state, probably as a proenzyme. Owren (1951) has also accepted the view that the factor is in a precursor state and has designated it proaccelerin. As Alexander *et al.* (1950) pointed out, the activity which is called serum Ac-globulin (and one can also include Owren's accelerin) is in all probability a mixture of plasma Ac-globulin or labile factor with S.P.C.A. The hypothesis that labile factor exists in blood in a precursor state and that when activated it becomes an accelerator is based on experimental observations which require reinterpretation.

The probable place at which labile factor enters the coagulation reaction was investigated by Quick and Stefanini (1950). They observed that little or no prothrombin consumption occurs when tissue thromboplastin (rabbit brain extract) is added to recalcified stored plasma, but that the addition of increasing amounts of labile factor to this system brought about corresponding increments of prothrombin consumption. This indicated that labile factor acts stoichiometrically and is essential in the utilization of extrinsic thromboplastin. Our observation that prothrombin consumption is very low in a patient with congenital deficiency of labile factor shows that the factor is also essential for the utilization of intrinsic thromboplastin. It can be concluded that labile factor enters at a late stage of the clotting reaction. It actually appears to be a thromboplastin co-factor, a term which Honorato (1947) suggested. Douglas (1956) has also concluded that the factor enters at a stage later than antihaemophilic globulin, Christmas factor, and platelets.

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THE BUCCAL MUCOSA IN ANAEMIA

BY

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The histological features of the buccal mucosa have been examined in 50 cases of iron-deficiency anaemia, 14 cases of megaloblastic anaemia, and 50 normal subjects. Differences between the anaemic and normal groups have been found and in some anaemic patients striking morphological changes have been seen. These might be due to a direct effect of iron or vitamin B_{12} deficiency on the metabolism of the epithelial cells.

The pathology of the buccal mucosa in anaemia is not fully understood, although symptoms related to the oral cavity, such as glossitis and stomatitis, are commonplace in severely anaemic patients. These symptoms may also be due to deficiency of iron or vitamin B_{12} in the absence of anaemia (Waldenström, 1938; Adams, 1957).

Material

Three groups of subjects were investigated.

(1) Fifty Cases of Chronic Iron-deficiency Anaemia.—No case with a haemoglobin concentration of 9 g. per 100 ml. or higher was included. The degree of anaemia is shown in the Table.

TABLE
DEGREE OF ANAEMIA IN PATIENTS INVESTIGATED

Haemoglobin (g. per 100 ml.)	3·0–3·9	4·0–4·9	5·0–5·9	6·0–6·9	7·0–7·9	8·0–8·9	Total
Iron-deficiency anaemia (cases)	1	1	4	15	17	12	50
Megaloblastic anaemia (cases)	0	3	2	6	2	1	14

In 32 cases the serum iron was less than 20 μg./100 ml., and in only three cases was it greater than 50 μg./100 ml.

An estimate of the duration of anaemia was made from the patient's history. Seven had had symptoms for only a few months. Thirty-three had a history of over a year, and in some cases many years. In 10 cases no estimate could be made of the duration of the condition.

Symptoms relating to the mouth or pharynx were present in 15 patients, and koilonychia was found in four. All these patients were estimated to have been anaemic for over a year. There was no relation between the presence of oropharyngeal symptoms and the degree of anaemia, but all these patients except one had serum iron levels below 20 μg./100 ml. Ten patients in this group complained of dysphagia, and further examination revealed the presence of a post-cricoid web in one case and a post-cricoid squamous carcinoma in another.

(2) Fourteen Cases of Megaloblastic Anaemia.—Twelve of these patients suffered from classical Addisonian pernicious anaemia, one from Addisonian anaemia associated with hypogammaglobulinaemia, and one from multiple jejunal diverticula. The degree of anaemia is shown in the Table. Serum vitamin B_{12} levels were estimated in the last two cases and in six of the others. The range obtained was 15–70 $\mu\mu$g./ml. (normal 140–600 $\mu\mu$g./ml.).

Only two patients in this group complained of oropharyngeal symptoms. Both included dysphagia amongst their complaints, but no radiological lesions were found.

(3) Fifty Non-anaemic Patients.—These patients were convalescing from minor medical or surgical conditions and apparently in good health.

None of the patients in any group suffered from a dermatological abnormality.

Methods

Specimens of the buccal mucosa were obtained by means of Wood's gastric suction biopsy tube. The orifice in the head of the tube was placed firmly but gently against the inside of the cheek between the first molars. A disc of epithelium, and usually some subepithelial tissue, about 2 mm. in diameter, was obtained and immediately fixed in

10% formol saline. The specimens were embedded in paraffin and orientated so as to obtain vertical sections through the mucosa. Sections were stained with haematoxylin and eosin. In a few instances, due either to obliquity of the section or to inadequate depth of the specimen, a full series of observations was not possible. Two biopsies were taken at different times from one patient with iron-deficiency anaemia.

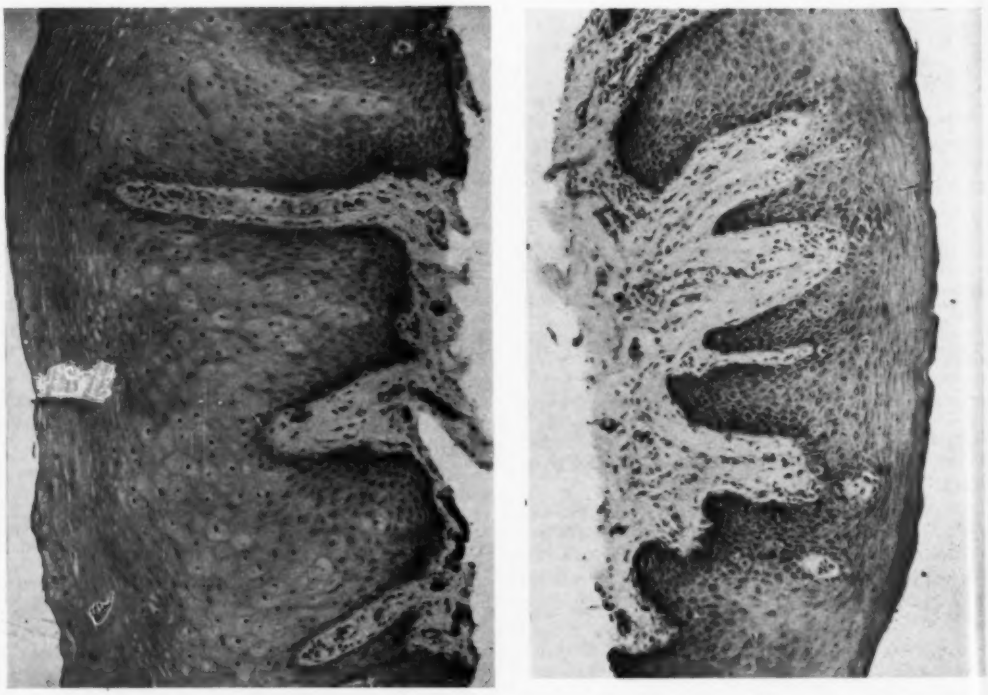
The biopsy procedure is almost painless and no local anaesthetic is necessary. Most patients consider it to cause less discomfort than a finger-prick. No complications have been discovered in more than 300 biopsies.

Histological Findings

The appearance of the normal buccal epithelium is somewhat variable. Fig. 1 shows two normal specimens and illustrates the extremes of thickness. Most of the biopsies from anaemic patients cannot be clearly differentiated from the normal, but certain features have a different incidence in the anaemic groups as a whole, and in a few cases there are obvious abnormalities. Individual characteristics of the buccal mucosa will be considered separately.

Epithelial Thickness. — Measurements were made of the maximum and minimum thickness of epithelium in each specimen. In the normal group some reduction in the maximum measurement was found in older subjects and appears to reflect a tendency for the interpapillary pegs to decrease in size with age. The maximum thickness of the epithelium fell below the normal range in nine out of 43 cases of iron-deficiency anaemia, and in three out of 14 cases of megaloblastic anaemia. In some cases thinning of the epithelium was marked (Fig. 2). The mean epithelial thickness of the anaemic groups as a whole was not significantly reduced.

Seven of the nine iron-deficient cases showing abnormal thinning had serum iron levels below 20 μg./100 ml.



a *b*

FIG. 1.—(*a*) Normal thick epithelium. (*b*) Normal thin epithelium. Haematoxylin and eosin × 100.

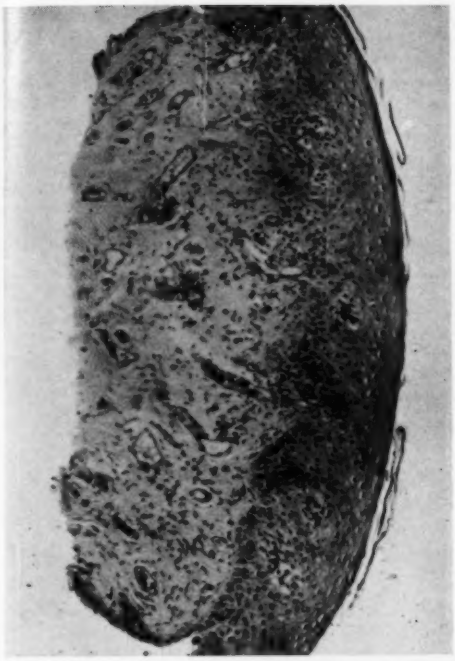


FIG. 2.—Epithelial atrophy in a case of iron-deficiency anaemia. Haematoxylin and eosin ×100.

	Maximum	Minimum
Normal ..	434 $\mu \pm 13{\cdot}9$ (280–655)	185 $\mu \pm$ 9·5 (85–350)
Iron-deficiency anaemia ..	413 $\mu \pm 25{\cdot}8$ (175–780)	186 $\mu \pm 13{\cdot}7$ (90–520)
Megaloblastic anaemia ..	360 $\mu \pm 29{\cdot}1$ (200–610)	199 $\mu \pm 21{\cdot}1$ (80–350)

Zone of Cornification.—The earliest morphological sign of cornification in the prickle cell layer of the epithelium is the sudden flattening of cells perpendicular to the surface and a tendency to pyknosis in their nuclei. The thickness of this flattened zone varies greatly between individual specimens, but there is no difference between the three groups.

	Mean Thickness
Normal	40 $\mu \pm 4{\cdot}9$ (0–110)
Iron-deficiency anaemia	38 $\mu \pm 4{\cdot}1$ (0–120)
Megaloblastic ,, ...	41 $\mu \pm 6{\cdot}1$ (5–90)

Keratin Formation.—Cornification of the buccal mucosa does not normally proceed to the production of fully formed keratin, and none was found in the control group of biopsies. In three cases from the iron-deficiency group mature keratin was seen (Fig. 3), and in one there were also areas of parakeratosis (Fig. 4). All three cases had serum iron levels below 20 μg./100 ml.

Keratohyaline Granules.—The relation of these granules to keratin formation is debatable. They were found in 37 of the normal group, in 40 of the iron-deficiency group, and in all the 14 cases of megaloblastic anaemia.

Basal Cell Mitoses.—The mitotic rate in the buccal epithelium varies considerably, but is higher than that found in the skin by Thuringer and Katsberg (1959), and unlike the skin there is no obvious increase in mitosis with age. The mean values for each group were:

	Mitoses per 1,000 Basal Cells
Normal	23 $\pm$ 2·3 (1–76)
Iron-deficiency anaemia	27 $\pm$ 2·9 (3–80)
Megaloblastic ,, ...	32 $\pm$ 7·2 (2–102)

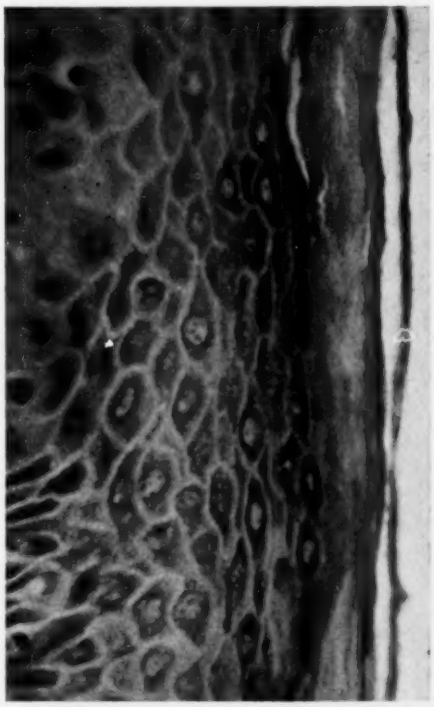


FIG. 3.—Keratin formation in a case of iron-deficiency anaemia. Haematoxylin and eosin ×440.

There is no significent difference between the groups.

Prickle Cell Mitoses.—Mitoses are not seen as frequently in the prickle cell layer as in the basal layer, and counts were not carried out. A note was made of the presence or absence of such mitotic cells in the sections of each specimen examined. They were seen in five (10%) of the normal group, 13 (25%) of the iron-deficiency group, and one (7%) of the megaloblastic group. The proportion of cases in which mitotic cells were seen is significantly higher in the iron-deficiency group than in the others. This does not seem to be related to the basal cell mitotic rate.

Binucleate Prickle Cells.—These were found in only four (8%) of the normal group, in nine (18%) of the iron-deficiency group, and in seven (43%) of the megaloblastic group (Fig. 5). They were seen at all levels of the epithelium, and their presence was not related to the number of basal cell mitoses or the presence of mitotic prickle cells.

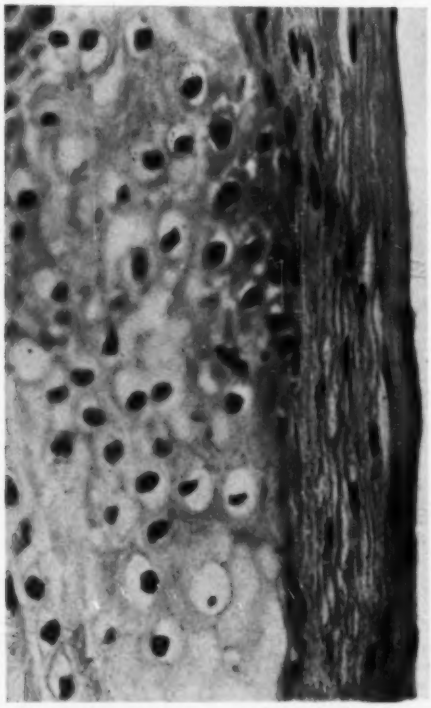


FIG. 4.—Parakeratosis in a case of iron-deficiency anaemia. Haematoxylin and eosin ×440.

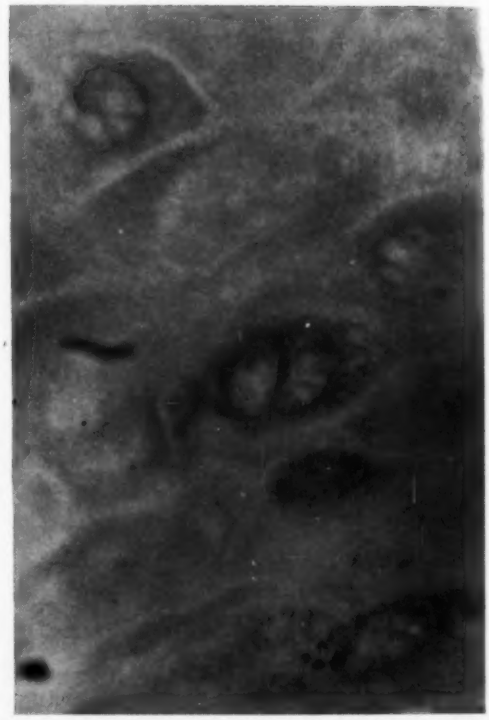


FIG. 5.—Binucleate prickle cell in a case of pernicious anaemia. Haematoxylin and eosin ×990.

Melanin.—This pigment may be found in 74% of buccal mucosae when staining by silver impregnation is carried out (Becker, 1927). It is not easily visible without this procedure except in a few cases. The presence of melanin, detectable without special staining, was sought in all specimens. In this way trace amounts were disregarded. It was found in the following proportion of cases:

	Basal Layer	Dermis
Normal	36%	38%
Iron-deficiency anaemia	24%	32%
,, ,, (patients with oropharyngeal symptoms)	7%	14%
Megaloblastic anaemia	12%	14%

The proportion of cases in which melanin can be seen in this way is significantly smaller than normal in those iron-deficient patients with symptoms related to the mouth or pharynx and in the megaloblastic anaemias.

Inflammatory Cells.—The presence of inflammatory cells in the epithelium and in the sub-

stantia propria was noted. When present they consisted mainly of lymphocytes, but sometimes a few plasma cells or polymorphs were also seen. When they were numerous the basal layer often became somewhat disorganized and poorly defined (Fig. 2). The proportion of each group showing evidence of inflammation was:

	Subepithelial	Intra-epithelial
Normal	22%	10%
Iron-deficiency anaemia	45%	12%
Megaloblastic ,, ...	43%	7%

The proportion of cases of iron-deficiency anaemia with evidence of subepithelial inflammation is significantly larger than in the normal group.

Discussion

The abnormalities of the buccal squamous epithelium which occur in iron-deficiency and the megaloblastic anaemias have been previously investigated mainly by the techniques of exfoliative cytology applied to gastric washings, saliva, or scrapings from the inside of the mouth. Graham and Rheault (1954) showed a generalized enlargement of squamous cells and their nuclei in pernicious anaemia and found some cells with giant nuclei. Binucleated cells were not uncommon. Similar changes have also been demonstrated in sprue (Gardner, 1956) and in subacute combined degeneration of the spinal cord unassociated with anaemia (Boen, Molhuysen, and Steenbergen, 1958). Boddington (1959) found that although these changes are most striking in megaloblastic anaemias they may also be seen, though to a lesser degree, in iron-deficiency anaemia. They are more common in patients with depapillation of the tongue and may sometimes be seen in patients with abnormal tongues not associated with anaemia. Jacobs (1959) has shown an increase of cornification in the squames from patients with chronic iron-deficiency anaemia.

The histological appearance of the complete oral mucosa in the anaemias has received scant attention, although the clinical picture of atrophy and thinning of the epithelium has long been recognized. Those observations which have been made are mainly from advanced cases of the "anaemia with dysphagia" syndrome. Paterson (1919), in his original description of "A Clinical Type of Dysphagia," mentions in passing that in the tongue and cheek there is a thinning of the epithelium and an infiltration of the underlying connective tissue. Suzman (1933), reporting a case of the "Paterson-Kelly" syndrome, describes thinning and keratinization of the epithelium of the tongue and oesophagus with round cell infiltration of the submucosa at both sites. McGee and Goodwin (1938), recording a similar case, state that the oesophageal mucosa was neither atrophied nor keratinized, but there was a chronic inflammatory reaction in the submucosa. They do not refer to the oral lesions. Savilahti (1946) describes keratinization of the oral mucosa near an angular fissure in a case of anaemia with dysphagia, and states that the epithelium was thin over the tongue, pharynx, and oesophagus. In the upper oesophagus he found the cells adjoining the basal layer to be in a state of rapid division. He also notes the presence of abnormally large nuclei at this site.

Thinning of the epithelium of the tongue in a case of pernicious anaemia has been noted by Taft, Hughes, and Wood (1958).

The present cases of iron-deficiency anaemia were unselected except for the exclusion of patients with a haemoglobin level of 9.0 g. per 100 ml. or above. Some show pathological thinning of the epithelium and gross abnormalities such as keratin formation and parakeratosis, but the majority are

FIG. 6.—Squamous carcinoma of the oesophagus in a case of iron-deficiency anaemia (same case as Fig. 2). Haematoxylin and eosin ×70.

not significantly different from the normal. Round-cell infiltration in the subepithelial connective tissue is common. The apparent increase in mitotic prickle cells may be a reflection of the crude methods of assessment. Oropharyngeal symptoms, such as sore tongue, sore mouth, and dysphagia, were not apparently related to the presence of any of these features. The only histological characteristic seen more commonly in patients with symptoms was a loss of melanin from the basal layer and the underlying tissue.

In the megaloblastic anaemias the interpapillary pegs also showed a tendency to atrophy, but this was not significant for the group as a whole. The presence of keratohyaline granules in all cases is perhaps surprising as mature keratin formation was not found at all. The presence of binucleate prickle cells was common in this group and loss of melanin was striking.

Depigmentation of the hair is known to occur in both pernicious and iron-deficiency anaemia, and vitiligo is also associated with these conditions. The loss of pigment from the mouth is probably part of a generalized disorder, the nature of which is unknown.

In three patients with iron-deficiency and one with pernicious anaemia complaining of dysphagia, biopsies were taken from the upper end of the oesophagus. No gross abnormality was found, but there was a mild degree of round cell infiltration under the epithelium in all the specimens. One patient with iron-deficiency anaemia who had had a sore tongue and angular stomatitis for nearly two years, and dysphagia for two months, was seen to have marked koilonychia. Biopsy of the oesophagus showed a well-differentiated squamous carcinoma arising from apparently normal epithelium (Fig. 6). The buccal epithelium, however, showed an atrophic stomatitis (Fig. 2). Cheli, Dodero, Celle, and Vassalotti (1959), who found atrophic changes in the oesophageal mucosa of seven out of 16 patients with iron-deficiency anaemia, saw no relation between this and depapillation of the tongue. It appears that not only are the pathological changes in the mouth not always reflected by the oesophageal mucosa, but that post-cricoid carcinoma does not necessarily arise as a sequel to these changes.

Conclusion

The main changes seen to occur in the buccal mucosa of some anaemic patients are a decrease in epithelial thickness and some loss of melanin pigment. Only the latter is apparently related to the presence of oral symptoms. In iron-deficiency anaemia keratin may be formed, and in the megaloblastic anaemias binucleated cells are often seen. These abnormalities are probably the result of multiple metabolic defects in the skin which may be the sequel of iron and vitamin B_{12} deficiency. The widely diverse processes of melanin formation, epidermal cell division, and cornification which are affected make a simple parallel between the mucous membrane changes and the bone marrow changes in anaemia (Gorz-Kardaszewicz, 1956) difficult to accept. It is unlikely that the changes observed are the direct result of anaemia, as they are not related to the haemoglobin concentration and they have been seen to occur in the absence of anaemia.

Specific deficiencies of iron-containing enzymes have been demonstrated in some tissues of iron-deficient animals (Beutler, 1957, 1959; Beutler and Blaisdell, 1960), and it is possible that similar defects in the metabolically active buccal mucosa may exist. A reduced concentration of iron in the finger-nails, an epidermal derivative, has been shown to exist in some iron-deficient patients (Jacobs and Jenkins, 1960). The part played by vitamin B_{12} in tissue metabolism is not clear, and the possibility that some of the tissue changes following iron deficiency may be due to a secondary lack of vitamin B_{12}, such as that demonstrated by Cox, Meynell, Gaddie, and Cooke (1959), should be considered.

I am most grateful to the medical staff of Paddington General and St. Charles' Hospitals for permission to obtain specimens from their patients; to Dr. R. R. Wilson and Dr. S. Shaw for their encouragement and criticism; and to Dr. D. L. Mollin for the vitamin B_{12} estimations. The Wood's tube was purchased by the Paddington General Hospital research fund.

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A RAPID "SIDE-ROOM" METHOD FOR THE DETERMINATION OF PLASMA FIBRINOGEN CONCENTRATION AS FIBRIN

BY

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A method is proposed for the rapid estimation of plasma fibrinogen concentration. The technique depends on the increase of optical density occurring when plasma clots. A kit is described which obviates the use of laboratory apparatus, so that the test may be performed in the ward side-room if a small centrifuge can be provided.

A calibration curve has been prepared for use with commercial opacity standards, and a measure of the error of the method has been obtained.

It would seem that when haemostatic function is in doubt, the fibrinogen concentration of plasma is more appropriately determined as material capable of clotting, i.e., as fibrin, than in terms of a precipitate thrown down by standard unphysiological conditions. While citrated plasma readily forms a clot on recalcification, methods previously suggested for the estimation of clot usually demand specialized equipment and are often time-consuming.

Rapid, simple methods recently proposed for fibrinogen determination have been based on unphysiological procedures, or have employed the reaction with thrombin to provide a more qualitative test. The use of this latter test is obviously important and is discussed below, but there still seems to be a place for a method for rapidly estimating clot with simple equipment.

The optical opacity of plasma increases markedly on coagulation. The rise in opacity bears a close relation to fibrin mass, and the conditions which govern this relation have received

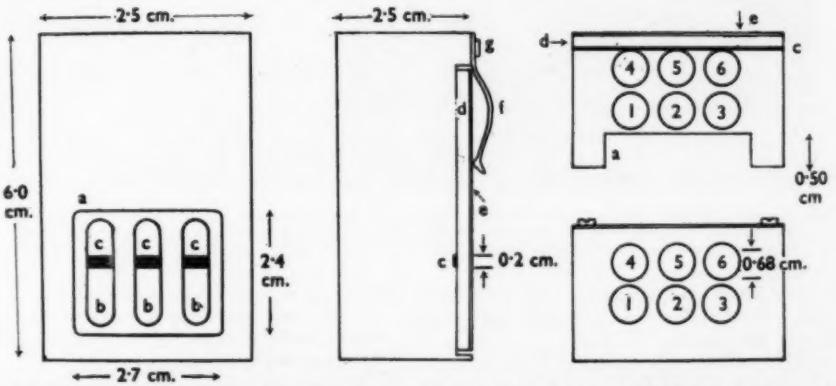

FIG. 1.—Diagram of the comparator block. The prototype was machined in ebonite, and the black line was drawn in Indian ink on a piece of glass cut from a shell-backed sheet such as is used for viewing by transmitted light the agglutination in the Race–Coombs test. The block is recessed at *a* over the viewing slits *b* through which is seen the black line *c* inscribed on the glass *d* with shell back *e*. The glass is held in position by the spring clip *f* bolted to the block at *g*.

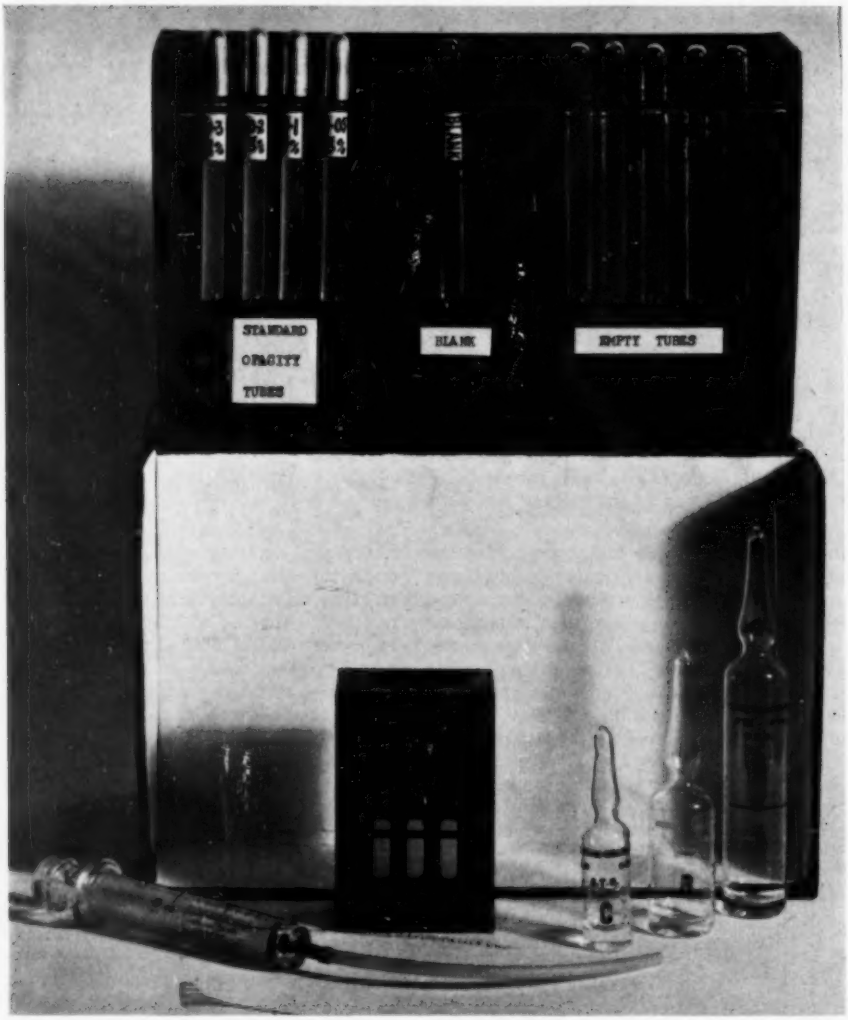

FIG. 2.—The side-room assembly. The ampoules contain measured volumes of the reagents required for a single test, and the opacity standards are calibrated in fibrinogen concentrations. If laboratory glassware is not available, the plasma can be measured by a syringe carrying a length of polythene catheter (as shown) or a wide-bore needle.

considerable attention (Nygaard, 1941; Burstein and Loeb, 1952). The method here proposed exploits this relationship.

Method

Principle.—Citrated plasma is recalcified and allowed to clot in a precipitin tube ($\frac{1}{4}$ in. × 4 in.). The opacity of the clot is determined by comparison with a series of Brown and Kirwan's (1915) standard opacity tubes which are commercially available. Colour correction is obtained by viewing the standard tubes through the same plasma similarly treated with saline in a two-row, six-hole comparator block.

To facilitate using in a side-room, the reagents may be prepared in ampoules containing the exact amounts required for each test; and the plasma may be manipulated with syringes if laboratory glassware is not available.

Equipment.—The following is required:

(1) A set of opacity standards, including a " blank " tube containing only the gelatin used to suspend the opaque material in the standards. It is suggested that for obstetric use it would be sufficient to provide four standards corresponding to 0.3 g.% (lower limit of normal late pregnancy), 0.2 g.% (lower limit of non-pregnant normal, i.e., presumptive haemostatic sufficiency), 0.1 and 0.05 g.% (representing moderate and severe deficiency respectively).

(2) A comparator block with six holes, to take $\frac{1}{4}$ in. tubes (17/64 in. drill) (Fig. 1).

(3) Empty $\frac{1}{4}$ in. × 4 in. glass tubes (three are required for a test).

(4) Flat-bottomed ampoules of reagents, containing (*a*) 0.5 ml. 3.8% sodium citrate solution in a 10 ml. ampoule, graduated at 5.0 ml.; the ampoule diameter should not exceed 5/8 in. so as to fit the test-tube-size metal bucket of a centrifuge; (*b*) 0.5 ml. 0.85% saline; (*c*) 0.5 ml. 0.025 M (0.28%) calcium chloride solution.

(5) Lengths of soft polythene catheter, about $3\frac{1}{2}$ in.; size 4 fits a " record " syringe nozzle.

These items are shown together in Fig. 2. In addition, the only piece of standard equipment required is a centrifuge to spin the citrated blood, since the test must be made on plasma.

Technique.—(1) Open one each of the ampoules marked (*a*), (*b*), and (*c*), having shaken down any solution held in the necks. Stand the ampoules upright on the bench.

(2) Fill (*a*) up to the mark with blood and mix by gentle inversion. Centrifuge the ampoule for 5 min. at about 3,000 r.p.m. (see that the upper part of the opened ampoule projects sufficiently from the centrifuge bucket for easy withdrawal).

(3) If laboratory glassware is not available, push a length of polythene tubing or a long, wide-bore needle on to the nozzle of a 1 ml. or 2 ml. syringe. Suck up 1.0 ml. of clear plasma and run 0.5 ml. into (*b*) and into (*c*). Mix the contents of (*b*) and apportion the liquid between two of the glass tubes by means of the syringe. Then immediately mix the contents of (*c*) and similarly transfer about half its contents to a third tube. The tubes filled from (*b*) are placed in holes 1 and 3 of the comparator and the tube filled from (*c*) in hole 5.

(4) Set the comparator aside for 10 to 20 min. for clotting to occur. (If the clotting tube can be incubated at 37° C., 5–10 min. will suffice.)

(5) Place the " blank " tube in hole 2 of the comparator, and, viewing through the slits in the front face, match the opacity seen through the central slit against standard tubes placed in holes 4 and 6 and seen through the side slits. It is helpful to consider together adjacent pairs of standard tubes, to define the opacity range which brackets that of the test plasma.

Notes.—(1) See that all glass tubes are quite clean over the lowermost inch of their length.

(2) Hold the comparator against a uniform diffuse white light when viewing. Some workers may prefer to match opacities by the degree of blurring of the black line, while others may find it easier to judge by the relative darkening of the bright ground. Strictly speaking, the value obtained will always be an underestimate, because of the dilution of the plasma with the citrate solution,

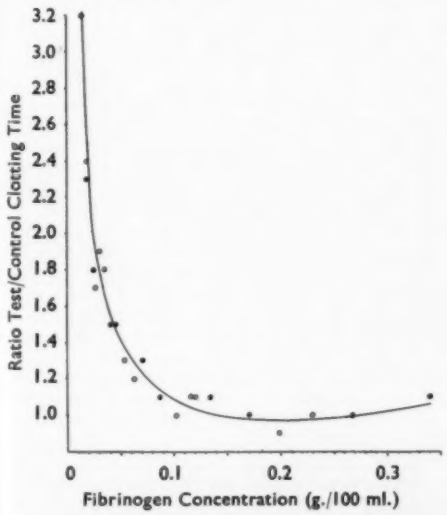

FIG. 3.—The relation between thrombin clotting time and fibrinogen concentration. The tests were made with a solution of human thrombin in M/80 calcium chloride solution, giving a control plasma clotting time of about 10 sec. when 2 volumes of thrombin solution were added to 1 volume of plasma. The plasma samples were those of Fig. 4.

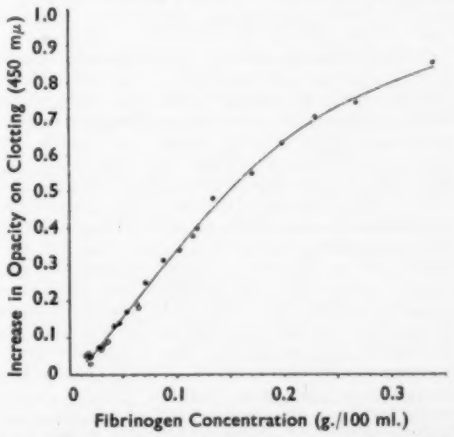

FIG. 4.—The relation between increase of plasma opacity on clotting and fibrinogen concentration. Measurements were made on 24 mixtures of whole and defibrinated plasmas prepared from normal and phenindione-treated subjects. Increase in opacity was obtained from the difference between the opacities (against water) of the recalcified, citrated plasma before and after clotting.

but the proportionate effect (which may be calculated in a given case from the haematocrit) is usually of such an order that the estimate is not less than 0.80 to 0.85 of the true value, an error which would be of small account under the proposed conditions of testing.

The quantitation of the method and an investigation of errors is given in the Appendix.

Relation Between Fibrinogen Concentration and Thrombin Clotting Time

One more point was investigated. Sharp, Howie, Biggs, and Methuen (1958) described three obstetrical cases in which the fibrinogen concentration had been reported normal but the thrombin clotting time was grossly prolonged. The significance of this finding is not yet clear, but it was thought of interest to provide a chart showing the relation between thrombin clotting time and the concentration of normal fibrinogen for comparison (Fig. 3). The readings were taken in the specimens providing the data for Fig. 4; the clotting times of the mixtures of whole and defibrinated plasmas were expressed as ratios of the clotting times of the corresponding untreated plasma pools.

We would like to thank our obstetrical colleagues for their encouragement in this investigation; and we are indebted to Dr. P. Armitage, of the Medical Research Council's Statistical Research Unit, for his advice on the investigation of the errors of the methods, and to Dr. A. J. Buller, of the Department of Physiology, St. Thomas's Hospital Medical School, for a discussion of the incremental sensitivity of the eye.

APPENDIX

Quantitative Basis

In developing the method, the relationship between opacity increase and fibrin mass was established by preparing from a number of pools of fresh, citrated human plasma several mixtures of whole and thrombin-defibrinated (Warner, Brinkhous, and Smith, 1936) plasma in differing proportions, and then measuring in each mixture both the opacity increase during clotting and the fibrin content (Ingram, 1952). The opacity measurements were made on a "unicam" S.P. 350 diffraction-grating spectrophotometer (with fixed slit-width), set at 450 mμ to obtain maximal dispersion. Plasma pools from both normal and phenindione-treated persons were examined, to detect systematic effects from at least a moderate coagulation defect. The results (Fig. 4) show a close correlation between the two series of measurements. There was no evidence of systematic differences between plasma pools or between plasmas from normal and phenindione-treated subjects.

Next, an aqueous suspension of the opaque material used in preparing the commercial Brown and Kirwan's standard opacity tubes was obtained by courtesy of the

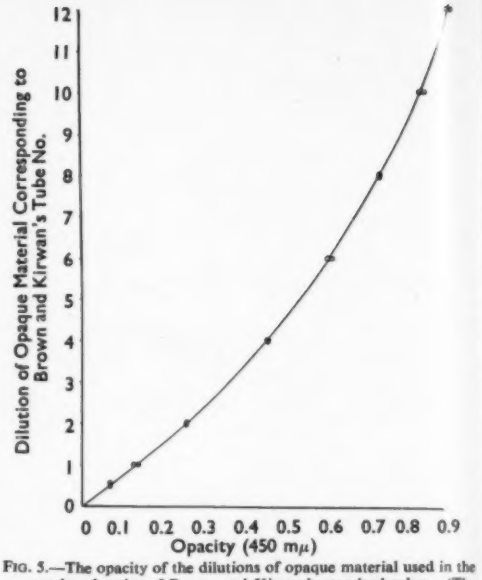

FIG. 5.—The opacity of the dilutions of opaque material used in the marketed series of Brown and Kirwan's standard tubes. (The numbers indicating the tube opacities are the numerators of the fractions, 12/12, 11/12, 10/12, . . .) The readings were taken under the same conditions as were employed for Fig. 3.

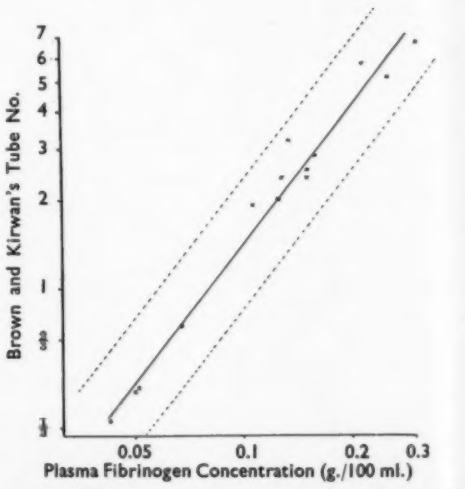

FIG. 6.—Calibration curve for Brown and Kirwan's standard opacity tubes (Wellcome Foundation Ltd.) in terms of fibrin content of clotted plasma. Fourteen different concentrations of fibrinogen in plasma were each examined in the comparator by six observers. The figure shows the mean readings for each fibrinogen concentration, and the ±2 S.D. limits for a single observer testing single samples of different plasmas on different occasions. The correlation coefficient for the 84 readings is +0·96 and the equation to the line is $Y = 52\ X^{1.8}$ where Y is the opacity of the standard tube in conventional numbering, and X is the fibrinogen concentration of the plasma in g./100 ml.

TABLE I

ANALYSIS OF VARIANCE OF 84 READINGS OF THE COMPARATOR WITH PLASMAS OF KNOWN FIBRINOGEN CONTENT

Contribution		Degrees of Freedom	Sums of Squares	Mean Square	Variance Ratios
1. *Between fibrinogen concentrations*		13			
Either (for differences between slopes of individual plasmas)	(i) Between slopes	6	9·40	1·57	n.s.
	(ii) Due to parallel lines	1	837·43		
	(iii) Between concentrations within plasmas	7	846·83		
	(iv) " plasmas	6	529·98		
Or (for differences between positions of individual plasmas)	(v) Deviation of plasma means from line having the " within plasmas " slope	6	28·57	4·76	3·84†
	(vi) Deviations within plasmas from parallel lines	6	9·40		
	(vii) Deviations from overall slope	12	37·97		
	(viii) Overall slope	1	1,338·85		
2. *Between observer pairs*		2			
	(i) Regression on skill	1	6·45	6·45	5·19†
	(ii) About regression	1	0·30		
3. *Fibrinogen concentration × observer pairs*		26	32·25	1·24 (2·18*)	1·00
4. *Error, within observer pairs*		42			
	(i) Systematic	3	5·93	1·98 (3·47*)	
	(ii) Residual	39	22·07	0·57 (1·00)	
	Total	83	1,443·82		

Seven plasma samples each provided two fibrinogen concentrations and each one was estimated by six observers.

The analysis was made in logs. to facilitate the computations involving the regression of comparator reading on fibrinogen concentration. The transformation had the incidental effect that the variability of the lower readings became rather greater than that of the higher readings

The error within observer pairs (4, i) showed no evidence of a regression on skill. The significant interaction (3) is thought to be due to circumstantial differences between different occasions of observation, such as differing intensities of lighting, and is therefore used to test the significance of the main effects.

Level of significant variance ratios: * 5%; † 1%; n.s., not significant at 5%.

Wellcome Foundation Ltd. Dilutions (10/12, 8/12, 6/12 . . .) were prepared corresponding to the usual series of tubes marketed and their opacities were measured under similar conditions. The results (Fig. 5) show that this range conveniently covers that of fibrinogen concentrations of clinical importance in obstetric emergencies.

The direct calibration of Brown and Kirwan's tubes in the comparator was made by visual comparison, as in ordinary use, with a series of mixtures of whole and defibrinated plasmas from seven individual citrated blood samples, so that each sample provided two differing mixtures. The fibrin contents of the mixtures were determined as before, and in each instance comparator readings were taken by six persons, who remained the same throughout the series, namely, the authors, two experienced laboratory workers who had not previously used this technique, and two laboratory secretaries, the three groups representing three degrees of skill with the method. The observers stated their score to the nearest standard tube, without interpolating. The paired mixtures from each plasma were examined concurrently, but each pair was handled on a different day. Eighty-four readings were thus obtained, six of each of 14 different concentrations of fibrinogen, over seven days.

The results are shown graphically in Fig. 6, in which the mean readings for the six observers are plotted against fibrinogen concentration to show the (significant) scatter of plasma means about the regression line calculated for all the 84 readings.

The analysis of variance is given in Table I. From this the various components of the error of a single reading may be obtained. For instance, taking the degree of variation found among observers and among plasmas and choosing an observer and a plasma sample at random on any day, if the fibrinogen concentration were read from the regression line of Fig. 6, the true fibrinogen concentration would be expected to lie between the limits:

Estimated value × 1.41 to estimated value × 1/1.41

in 19 out of 20 trials. If, however, the sources of variation are considered separately, the corresponding limits are given in Table II, where they are computed so as to provide a test for the significance of the difference between two readings, since this is likely to be a more difficult assessment than the simple detection of abnormality.

It is clear from the analysis that there was little discrepancy between observers in matching the appro-

TABLE II

ESTIMATES OF ERROR OF METHOD EXPRESSED AS A TEST FOR SIGNIFICANCE OF DIFFERENCE BETWEEN TWO INDIVIDUAL RESULTS UNDER VARIOUS CONDITIONS (95% CONFIDENCE LEVEL)

Conditions	Critical Ratio
1. Different observers taking single readings on different plasmas on different occasions	1·61
2. The same observer taking single readings on different plasmas on different occasions	1·57
3. Different observers taking single readings on different occasions on the same plasma	1·53
4. The same observer taking single readings on different occasions on the same plasma	1·49

To be significantly different, the two results must differ by a ratio greater than the critical value tabulated. Thus, suppose an observer obtains a reading of 0·10 g./100 ml. on a given patient, the same observer then obtains a subsequent reading of, say, 0·18 g.'100 ml. Can he confidently say that a rise in the fibrinogen concentration has occurred? Yes, because the conditions are those of (4) in the Table, assuming that other properties of the patient's plasma, such as the colour, are unaltered: the ratio between his readings is 1·8, which exceeds the tabulated critical ratio of 1·49.

priate turbidity standard to given sample of clotted plasma.

On the basis suggested above, it may be estimated from Fig. 6 that the four turbidity standards to be provided should have optical densities corresponding to about 7.6 units (≏ 0.3 g./100 ml.), 4.1 units (≏0.2 g./100 ml.), 1.3 units (≏0.1 g.× 100 ml.), and 0.5 units (≏0.05 g./100 ml.) on the conventional Brown and Kirwan scale used in preparing the standards commercially. The lower three of these fibrinogen values form a two-fold dilution series, so that the ratios between them clearly exceed the highest critical ratio given in Table II. This means that, with these four proposed standard tubes, it is very unlikely that experimental error would lead to an erroneous match in the pathological range.

Discussion

The analysis of variance (Table I) shows four significant variance ratios. Taking first the observer effects: (4, i), the systematic error within observer pairs, simply means that the readings of each individual observer were on the average distinguishable from those of his fellow; and (2, i), the regression on skill, reflects the finding that less " skilled " observers tended to record higher readings than the more " skilled "; but the whole of the observer variation amounts only to an S.D. range of 1.06× to 1/1.06× a given fibrinogen reading. The variation ascribed to different plasma samples (1, v) represents a corresponding S.D. range of 1.08× to 1/1.08×; variation from different occasions of testing (representing the interaction, 3) yields an S.D. range of 1.09× to 1/1.09× ; and the residual error mean square, (4, ii), gives a range of 1.12× to 1/12× for the random variation of a single reading. Thus systematic differences between observers, plasma samples, or occasions of testing are likely to be smaller than the random error.

There is one point of general interest which emerges from a comparison of Figs. 4 and 6. When Fig. 4 (the spectrophotometer readings) is replotted in log.-log. transformation, the slope of the regression of optical density on fibrinogen concentration is, as would be expected, near unity (the fit is linear except at the upper end) whereas the log.-log. regression obtained with the eye data of Fig. 6 has a significantly higher slope ($b = 1.57$). This might be related to the yellow-green colour of plasma, since it is known that the incremental sensitivity of the eye is generally non-linear (Barlow, 1958).

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DIAGNOSTIC DIFFICULTIES IN MILD HAEMOPHILIA AND CHRISTMAS DISEASE

BY

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Two cases of haemorrhagic diathesis are described, where the laboratory diagnosis was confused owing to previous blood transfusions.

Without the help of investigating the relatives, the nephew of Case 1, the haemophiliac, and the uncle of Case 2, with Christmas disease, there would have been considerable delay in reaching the diagnosis and so instituting the correct treatment.

Ordinarily the laboratory diagnosis of classical haemophilia (haemophilia A) and Christmas disease (haemophilia B, plasma thromboplastin component deficiency) is easy using the thromboplastin generation test as described by Biggs and Douglas (1953). Nevertheless difficulty arises when the patient mildly deficient in anti-haemophilic globulin or Christmas factor is transfused following haemorrhage.

The concentration of the deficient factor may be raised for a variable period above 30% with fresh whole blood or plasma, a level capable of producing a normal result with the thromboplastin generation test. The length of this interval is dependent on a number of factors which include the initial anti-haemophilic globulin level of the patient (between 5 and 25% in the mildly affected), the anti-haemophilic globulin content of transfused blood or plasma, the half-life of anti-haemophilic globulin (eight to ten hours), and the increased rate of utilization associated with haemorrhage. These comments are even more applicable in mild Christmas disease since the clotting factor is more stable and may last in the circulation for several days.

In hospital practice this difficulty in distinguishing abnormalities of mild degree causes therapeutic problems. To clarify the diagnosis repeated quantitative assays would be of value, but all methods are time consuming, some require special reagents, and others a constant source of the same plasma as a reference standard. Also the time necessary for the degradation of transfused anti-haemophilic globulin or Christmas factor will delay correct treatment; often it is not possible to postpone transfusion in order to permit the laboratory to make the correct diagnosis.

Although Hicks and Pitney's screening test (1957) is said to be more sensitive than the standard thromboplastin generation test, it does not differentiate between haemophilia and Christmas disease. It is now accepted that the degree of deficiency of the clotting factor in haemophilia and in Christmas disease is the same in the affected members of a family, i.e., if there is 5% or less anti-haemophilic globulin all will have the same concentration and be severe haemophiliacs. In between 5 and 25% the condition is considered mild and all affected members have mild haemophilia. Classical haemophilia and Christmas disease are inherited as sex-linked Mendelian recessives. With a carrier mother the daughters have a 50% chance of being carriers, and the sons the same chance of being bleeders. With a haemophiliac father all daughters will be carriers and the sons normal, i.e., neither carriers nor sufferers. With this in mind the relevant members of a family may be investigated by a planned approach.

The purpose of this short paper is to demonstrate the value of investigating the relatives of such patients.

Case Reports

Case 1.—Mr. G. C., aged 64, a blacksmith, was admitted to another hospital on October 31, 1955, complaining of two attacks of painless haematuria, one 18 months and the other nine days before admission. He had been in fairly good health and gave no history of excessive bleeding.

Examination revealed an enlarged prostate; biochemical investigations proved satisfactory and a prostatectomy was performed. Eleven days after, on November 21, the suprapubic wound was found to be leaking urine and the bladder was distended with large

blood clots. These were removed, but the condition recurred a week later. The patient had been given 4 pints of blood during the first operation and a further 6 pints during the following 20 days.

He was admitted to the General Hospital, Birmingham, on November 30, 1955, for further investigations after having a total of 10 pints of blood, 2 of these on the day before admission. On close questioning, he admitted that he bled excessively following dental extractions, and that his nephew (a sister's son) was similarly affected. Haematological investigations on admission had revealed no abnormality, when the haemoglobin was 81% (12.0 g.%), red blood cells 3,980,000 per c.mm., white blood cells 5,600 (normal differential), platelets 370,000 per c.mm., Hess's test negative, bleeding time (Ivy's method) 1½ minutes, clotting time (37° C., Lee and White) 8½ minutes, prothrombin level (Quick's one-stage method) 100%, clot retraction normal. The thromboplastin generation test gave a normal result (Table I).

TABLE I
FIRST THROMBOPLASTIN GENERATION TEST ON CASE I

Reagents	Incubation Time (min.)					
	1	2	3	4	5	6
Using normal platelets						
(1) Normal serum and normal adsorbed plasma ..	86	53	14	13	13	13
(2) Patient's serum and patient's adsorbed plasma ..	75	37	14	13	13	13
(3) Normal serum and patient's adsorbed plasma ..	84	50	16	15	14	14
(4) Patient's serum and normal adsorbed plasma ..	70	16	12	13	12	13
Using patient's platelets						
Normal serum and normal adsorbed plasma	62	34	15	13	13	12

Two days later the haemoglobin fell to 68% (10.1 g.%). The suprapubic wound was still leaking, so the bladder was reopened and again found to be distended with clotted blood; no bleeding point was discovered. Continuous irrigation of the bladder was begun and 3 pints of stored blood transfused. Four days later a further 3 pints were given and on the following day all bleeding ceased only to restart after an interval of three days. A repeat thromboplastin generation test taken five days following the last blood transfusion showed a definite pattern of mild haemophilia (Table II). Unfortunately before this abnormal result could be confirmed, the patient's general condition deteriorated rapidly and a cystoscopy was performed. Blood was seen coming from the right ureteric orifice and a right-sided nephrectomy was carried out. After this, bleeding occurred into the nephrectomy wound and was controlled by the application of fresh pork fat. He was given a further transfusion of 4 pints of blood and improved. Macroscopic examination of the kidney revealed no bleeding point; sections showed a moderate degree of pyelonephritis.

TABLE II
SECOND THROMBOPLASTIN GENERATION TEST ON CASE I

Reagents	Incubation Time (min.)					
	1	2	3	4	5	6
Using normal platelets						
(1) Normal serum and normal adsorbed plasma ..	45	20	15	16	15	15
(2) Patient's serum and patient's adsorbed plasma	49	32	27	26	20	22
(3) Normal serum and patient's adsorbed plasma ..	50	35	25	25	25	28
(4) Patient's serum and normal adsorbed plasma ..	45	15	15	16	16	15
Using patient's platelets						
Normal serum and normal adsorbed plasma	52	18	15	15	13	15

On December 20 investigations of the patient's nephew (A. F.), who had previously refused to come to hospital, showed that he bruised easily, bled excessively after dental extractions, and had had haemorrhage into the joints as a child. The result of the thromboplastin generation test showed that he was a mild haemophiliac (Table III). The third thromboplastin generation test on the patient, seven days after his last transfusion, then confirmed the anti-haemophilic globulin deficiency (Table IV).

TABLE III
THROMBOPLASTIN GENERATION TEST ON NEPHEW OF CASE I

Reagents	Incubation Time (min.)					
	1	2	3	4	5	6
Using normal platelets						
(1) Normal serum and normal adsorbed plasma ..	47	15	15	15	16	15
(2) Patient's serum and patient's adsorbed plasma	57	37	29	37	39	28
(3) Normal serum and patient's adsorbed plasma ..	47	38	40	46	38	45
(4) Patient's serum and normal adsorbed plasma ..	35	19	18	15	15	15
Using patient's platelets						
Normal serum and normal adsorbed plasma	30	17	15	14	15	15

The patient was then treated as a case of haemophilia with transfusions of fresh blood and plasma. No concentrated anti-haemophilic globulin

TABLE IV
THIRD THROMBOPLASTIN GENERATION TEST ON CASE I

Reagents	Incubation Time (min.)					
	1	2	3	4	5	6
Using normal platelets						
(1) Normal serum and normal adsorbed plasma ..	32	14	15	17	15	15
(2) Patient's serum and patient's adsorbed plasma	45	25	20	19	20	19
(3) Normal serum and patient's adsorbed plasma ..	43	23	24	24	21	28
(4) Patient's serum and normal adsorbed plasma ..	35	20	16	15	15	15
Using patient's platelets						
Normal serum and normal adsorbed plasma	48	17	15	14	13	15

of human or animal origin was available at that time. Despite antibiotic treatment, he developed bronchopneumonia and died on January 9, 1956. At necropsy the remaining kidney showed pyelonephritis.

Case 2.—Mr. G. P., aged 26, a storekeeper, was admitted to another hospital on October 26, 1959, after a haematemesis. Because of a history of a tendency to bleed easily he was transferred to the General Hospital, Birmingham, three days later for further investigation after the transfusion of 9 pints of blood. He had always bruised readily and bled excessively after dental extractions. At 12 years of age he was transfused with 4 pints of blood following the extraction of one tooth. He had had a haematemesis and melaena three years before for which no treatment was given. He had a married sister with no children and one unmarried brother, who were quite normal. However, an uncle, his mother's brother, was said to bleed excessively. Laboratory investigations of the patient showed that the haemoglobin was 71% (10.6 g.%), white blood cells 7,400 per c.mm., platelets 104,000 per c.mm.; Hess's test, prothrombin level, and clot retraction were normal. The bleeding time was three minutes at 37° C. (Lee and White's method). A thromboplastin generation test was indecisive (Table V) and the

TABLE V
THROMBOPLASTIN GENERATION TEST ON CASE 2

Reagents	Incubation Time (min.)					
	1	2	3	4	5	6
Using normal platelets						
(1) Normal serum and normal adsorbed plasma ..	23	11	14	16	12	13
(2) Patient's serum and patient's adsorbed plasma	23	12	13	16	14	16
(3) Normal serum and patient's adsorbed plasma ..	13	10	12	10	13	13
(4) Patient's serum and normal adsorbed plasma ..	24	17	19	18	18	22
Using patient's platelets						
Normal serum and normal adsorbed plasma	21	15	13	14	13	12

repeat gave a similar abnormal but non-diagnostic result. It was thought that he had mild Christmas disease, and that the thromboplastin generation test did not give a typical pattern because of the previous blood transfusion.

Investigations of his uncle, aged 60, showed that he too had bled excessively following dental extractions, usually necessitating plugging. Haematological investigations were quite negative, but a thromboplastin generation test revealed the presence of mild Christmas disease (Table VI). It was assumed that

TABLE VI
THROMBOPLASTIN GENERATION TEST ON UNCLE OF CASE 2

Reagents	Incubation Time (min.)					
	1	2	3	4	5	6
Using normal platelets						
(1) Normal serum and normal adsorbed plasma ..	60	15	9	10	9	8
(2) Patient's serum and patient's adsorbed plasma	70	19	16	20	16	15
(3) Normal serum and patient's adsorbed plasma ..	10	7	10	10	8	9
(4) Patient's serum and normal adsorbed plasma ..	40	20	25	24	23	28
Using patient's platelets						
Normal serum and normal adsorbed plasma	24	14	11	10	10	9

the patient G. P. was similarly affected, and he was treated by transfusing 7 pints of blood. He made a rapid recovery and was sent home on November 9. Two weeks later a repeat thromboplastin generation

TABLE VII
REPEAT THROMBOPLASTIN GENERATION TEST ON CASE 2

Reagents	Incubation Time (min.)					
	1	2	3	4	5	6
Using normal platelets						
(1) Normal serum and normal adsorbed plasma ..	90	60	16	13	15	14
(2) Patient's serum and patient's adsorbed plasma	120	80	35	33	27	28
(3) Normal serum and patient's adsorbed plasma ..	90	62	20	15	15	14
(4) Patient's serum and normal adsorbed plasma ..	95	52	37	32	30	29
Using patient's p ateiets						
Normal serum and normal adsorbed plasma ..	20	15	14	13	12	12

test clearly confirmed that he was a case of Christmas disease (Table VII). A barium meal as an out-patient showed a duodenal ulcer; he was placed on an ulcer regime and up to date is well.

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SERUM HAPTOGLOBINS IN HAEMOLYTIC STATES

BY

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Serum haptoglobin levels have been studied in patients with a number of haemolytic disorders.

In general, serum haptoglobins were low in patients with increased haemolysis. Values were reduced irrespective of whether haemolysis occurred intravascularly or extravascularly.

The reduction was related to the amount of haemoglobin destroyed per day.

Serum haptoglobins may be raised in certain neoplasms and infections and during adrenocortical steroid hormone therapy. A rise in haptoglobins due to one of these factors may mask a fall due to haemolysis. Thus a normal or raised serum haptoglobin does not necessarily exclude a haemolytic process.

It is now well recognized that the serum haptoglobins may be reduced in haemolytic states (Chevallier and Wolpé, 1945 ; Nosslin and Nyman, 1958 ; Allison, 1958 ; Neale, Aber, and Northam, 1958). The determination of haptoglobins in serum is thus of potential value in the detection of haemolysis. The purpose of this paper is to record serum haptoglobin levels in a number of different types of haemolytic disorders, to assess the relation of rate and site of haemolysis to the serum haptoglobin levels, and to examine the value of this estimation as an aid in the diagnosis of haemolytic states.

Methods

Serum haptoglobins were measured by a colorimetric procedure (Owen, Better, and Hoban, 1960a). The results have been expressed in terms of methaemoglobin-binding capacity (mg./100 ml.). This method gives a mean normal value (n=150) of 91 mg./100 ml. with a range (±2 S.D.) of 11 to 172 mg./100 ml.

Haemoglobin was measured photometrically as oxyhaemoglobin. Reticulocytes were stained with cresyl blue (Dacie, 1956). Erythrocyte counts were not performed, so that absolute reticulocyte counts could not be computed. As an approximate absolute index of reticulocytosis, the "reticulocyte haemoglobin" was calculated by multiplying the haemoglobin concentration by the fraction of erythrocytes present as reticulocytes (cf. Giblett, Coleman, Pirzio-Biroli, Donohue, Motulsky, and Finch, 1956). The normal reticulocyte haemoglobin was taken as 0.1–0.3 g./100 ml. Erythrocyte survival and blood volume studies were carried out using chromium-51 as previously described (Crawford and de Gruchy, 1958); data were not corrected for the loss of chromium by elution from erythrocytes. An index of peripheral haemoglobin destruction was calculated by multiplying the fraction of erythrocytes apparently destroyed daily $\left[\frac{0.69}{\text{chromium half-life}}\right]$ by the circulating haemoglobin (Veall and Vetter, 1958). The normal value for this index was taken, in terms of haemoglobin, to be 250 mg./kg./day (normal chromium half-life=28 days; circulating haemoglobin=10 g./kg.). Since the chromium half-life is less than the true half-life, the above formula overestimates peripheral haemoglobin destruction. Nevertheless values in different persons are still comparable.

Serum bilirubin was determined using the method of O'Hagan, Hamilton, Le Breton, and Shaw (1957). In some instances in which serum bilirubin was not measured, the colour of the plasma was recorded. Serum vitamin B_{12} was measured according to the method of Hutner, Bach, and Ross (1956).

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Patients Studied

The conditions studied were classified as follows:

Congenital Haemolytic Disease (10 Cases).—Four patients (Cases 1–4) had hereditary spherocytosis, four (Cases 5–8) had non-spherocytic congenital haemolytic anaemia, one (Case 9) had thalassaemia, and one (Case 10) had thalassaemia-sickle cell disease. One patient with hereditary spherocytosis (Case 4) had a megaloblastic marrow presumably due to folic acid deficiency. His serum vitamin B_{12} was 300 $\mu\mu$g./ml.

Paroxysmal Nocturnal Haemoglobinuria (One Case).

Chemical Haemolytic Disease (Seven Cases).—Two patients (Cases 12, 13) developed haemolytic anaemia following the administration of phenacetin, and another (Case 14) an acute haemolytic anaemia probably due either to phenylbutazone or dry cleaning solvents. Cases 13 and 14 showed methaemoglobinaemia; in Case 12 spectroscopic examination was not performed. Haemolysis ceased in all patients after the toxic agents were withdrawn and in two (Cases 12, 14) adrenocortical steroids were administered. Four other patients (Cases 15–18) had sulphaemoglobinaemia without evidence of haemolysis.

Disseminated Lupus Erythematosus (Six Cases).—In five of these cases the diagnosis was confirmed by the demonstration of L.E. cells; in the other the diagnosis was based on clinical features alone.

Leukaemia, Myelosclerosis, and Malignant Lymphomas (19 Cases).—The diagnosis was established by blood and marrow examinations or by histological examination of biopsy specimens.

Results

Congenital Haemolytic Disease.—The serum haptoglobins were low in all patients with untreated congenital haemolytic disease (Table I). Not all of the patients in this group were anaemic at the time the serum haptoglobins were determined, although they all showed evidence of increased haemoglobin destruction in the form of increased serum bilirubin and reticulocyte-haemoglobin levels.

Paroxysmal Nocturnal Haemoglobinuria.—This patient was anaemic and had a low serum haptoglobin level although the serum bilirubin was not increased.

Chemical Haemolytic Anaemia.—The serum haptoglobin levels were reduced in all three patients during the acute haemolytic episode. In Cases 12 and 14 haptoglobin levels returned to normal one month after the acute episode, by which time the haemoglobin value, reticulocyte count, and the serum bilirubin had returned to normal. In patients with sulphaemoglobinaemia without evidence of haemolysis, serum haptoglobins were low-normal in three (Cases 15–17) and high-normal in the fourth.

Disseminated Lupus Erythematosus. — In patients with this disorder the results were variable (Table III). Values were low in three patients (Cases 22–24) who showed evidence of haemolysis in that they were anaemic with a raised reticulocyte count, an increased serum bilirubin, and

TABLE I

SERUM HAPTOGLOBINS IN PATIENTS WITH CONGENITAL HAEMOLYTIC DISEASE

Case No.	Diagnosis	Sex	Age	Haemoglobin (g./100 ml.)	Reticulocyte-haemoglobin (g./100 ml.)	Bilirubin (mg./100 ml.)	Haptoglobin (mg./100 ml.)
1	Hereditary spherocytosis	M	21	14·6	0·6	1·9	<10
2	,, ,,	M	18	16·9	2·7	2·4	<10
3	,, ,,	F	29	10·9	1·7	1·6	19
4	,, ,,	M	64	6·5	0·4	1·5	<10
5	Non-spherocytic congenital haemolytic anaemia	F	9	3·7	0·4	1·7	11
6	,, ,,	M	43	6·2	1·6	Plasma jaundiced	11
7	,, ,,	M	45	12·0	—	2·5	<10
8	,, ,,	M	9	7·4	2·2	2·0	15
9	Thalassaemia	M	23	7·9	0·3	2·0	<10
10	,, -sickle cell disease	M	2	7·7	—	—	12

TABLE II

SERUM HAPTOGLOBINS IN PATIENTS WITH PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA (CASE 11), CHEMICAL HAEMOLYTIC ANAEMIA (CASES 12–14), AND SULPHAEMOGLOBINAEMIA (CASES 15–18)

Case	Sex	Age	Date	Haemoglobin (g./100 ml.)	Reticulocyte-haemoglobin (g./100 ml.)	Bilirubin (mg./100 ml.)	Haptoglobin (mg./100 ml.)
11	F	46	—	9·6	—	Plasma not jaundiced	14
12	M	23	23/10	6·3	1·9	,, jaundiced	<10
			24/11	13·7	—	,, not jaundiced	93
13	F	38	—	11·5	2·8	1·0	<10
14	F	45	5/11	5·3	0·5	—	<10
			10/11	12·9 after transfusion	1·0	2·0	16
				15·7	0·2	0·3	69
15	M	73	—	13·0	0·2	Plasma not jaundiced	48
16	F	76	—	12·1	—	,, ,, ,,	39
17	F	75	—	—	—	,, ,, ,,	42
18	F	84	—	—	—	,, ,, ,,	170

TABLE III
SERUM HAPTOGLOBINS IN PATIENTS WITH DISSEMINATED LUPUS ERYTHEMATOSUS

Case No.	Sex	Age	Date	Haemoglobin (g./100 ml.)	Reticulocyte-haemoglobin (g./100 ml.)	Bilirubin (mg./100 ml.)	Haptoglobin (mg./100 ml.)
19	F	60	—	11·1	0·1	0·5	250
20	F	17	—	12·1	0·2	0·3	144
21	M	55	—	12·0	0·2	Plasma not jaundiced	250
22	F	20	—	11·3	—	2·5	< 10
23	M	38	—	8·4	—	Plasma jaundiced	< 10
24	F	47	7/7/59	7·2	0·1	3·7	< 10
			16/7/59*	10·3	0·7	0·8	< 10
			27/10/59*	14·2	0·7	—	32
			19/1/60*	13·2	—	1·2	68
			21/2/60*	14·1	0·6	1·2	115

* On adrenocortical steroid therapy.

TABLE IV
SERUM HAPTOGLOBINS IN PATIENTS WITH MYELOSCLEROSIS, LEUKAEMIA, OR MALIGNANT LYMPHOMAS

Case No.	Diagnosis	Sex	Age	Haemoglobin (g./100 ml.)	Reticulocyte-haemoglobin (g./100 ml.)	Bilirubin (mg./100 ml.)	Haptoglobin (mg./100 ml.)
25	Myelosclerosis	F	68	8·5	0·1	0·2	10
26	"	M	61	5·5	0·1	0·4	125
27	"	F	64	8·0	0·2	Plasma not jaundiced	255
28	"	M	69	15·6	0·6	Plasma not jaundiced	27
29	Acute monocytic leukaemia	F	52	7·3	0·1	" " "	73
30	" " "	F	62	8·3	0·1	0·8 "	360
31	Chronic lymphatic leukaemia	F	59	5·6	0·2	Plasma not jaundiced	71
32	" " "	F	48	8·2	0·3	0·6	40
33	" " "	M	48	7·1	0·1	0·5	113
34	" " "	M	61	6·6	0·1	—	58
35	Acute myeloid leukaemia	M	46	9·1	0·1	—	204
36	" " "	M	50	7·8	0·9	0·5	510
37	Chronic " "	M	33	12·5	0·1	—	180
38	" " "	M	72	8·8	—	0·2	49
39	" " "	F	57	7·0	—	0·4	250
40	Lymphosarcoma	F	66	13·0	0·1	Plasma not jaundiced	60
41	"	M	32	8·3	0·1	" " "	354
42	Reticulum cell sarcoma	F	48	11·0	0·1	" " 0·2 "	350
43	" " "	M	73	11·6	0·2		248

an excess urobilinogen in the urine. In one of these patients (Case 24), who was given prednisolone, the serum haptoglobin slowly increased over seven months to reach the mean normal level in spite of continuing haemolysis as evidenced by raised reticulocyte haemoglobin and bilirubin levels. In the other three patients with no evidence of haemolysis the serum haptoglobins were either normal or high.

Myelosclerosis, Leukaemia, and Malignant Lymphomas.—With one exception the serum haptoglobins in patients with these disorders were either normal or increased (Table IV). The exception was a patient with myelosclerosis who was anaemic; she showed no clinical evidence of haemolysis, but her chromium T/½ was moderately reduced. Most of the other patients were anaemic, but none had elevated serum bilirubin levels and in only two was the reticulocyte haemoglobin raised. In these patients the haptoglobin levels were low-normal and high, respectively.

Haemoglobin Destruction Rates.—These were calculated in some patients (Table V). With few exceptions haptoglobin values tended to be low in those patients with high haemoglobin destruction rates and high serum bilirubin levels.

One patient (Case 4) had a peripheral haemoglobin destruction rate only slightly elevated although the serum bilirubin was 1.5 mg./100 ml.

TABLE V
SERUM HAPTOGLOBIN AND PERIPHERAL HAEMOGLOBIN DESTRUCTION

Case* No.	Haemoglobin (g./100 ml.)	Chromium Half-life (days)	Peripheral "Haemoglobin Destruction" (mg./kg./day)	Bilirubin (mg./100 ml.)	Haptoglobin (mg./100 ml.)
2	16·9	6	1,430	2·4	< 10
3	10·9	8	730	1·6	19
1	14·6	13	590	1·9	< 10
4	6·5	9	370	1·5	< 10
27	8·0	16	310	Plasma not jaundiced	90
32	8·2	17	310	"	60
31	5·6	14	300	0·6	40
25	8·5	18	280	0·2	< 10
42	11·0	20	210	Plasma not jaundiced	350
27	8·3	23	180	"	120
34	7·9	25	180	"	58
33	4·2	17	170	0·5	113

* Cases have been arranged in order of decreasing peripheral haemoglobin destruction.

However, this patient had a megaloblastic marrow, so that it is possible that haemolysis of immature cells was causing a raised bilirubin and a low haptoglobin level without the peripheral haemoglobin destruction rate being greatly affected (Owen, Carew, Cowling, Hoban, and Smith, 1960b).

Effect of Splenectomy.—In four cases serum haptoglobins were measured before and after splenectomy (Table VI). In three cases (all hereditary spherocytosis) the serum haptoglobins were low before the operation while the reticulocyte-haemoglobin and the serum bilirubin levels were increased. After operation, all three parameters returned to normal values. In the other patient with myelosclerosis all three parameters were within normal limits before and after the operation.

TABLE VI
EFFECT OF SPLENECTOMY ON SERUM HAPTOGLOBINS

Case No.	Time Relative to Splenectomy	Haemoglobin (g./100 ml.)	Reticulocyte-haemoglobin (g./100 ml.)	Bilirubin (mg./100 ml.)	Haptoglobin (mg./100 ml.)
1	Before	14·6	0·6	1·9	<10
	After	15·9	0·2	Plasma not jaundiced	60
3	Before	10·9	1·7	1·6	19
	,,	12·6	2·4	1·7	12
	+2 days	12·8	1·0	—	11
	+5 ,,	12·7	0·3	Plasma not jaundiced	22
	+13 ,,	12·8	0·1	0·2	120
	+22 ,,	13·9	0·2	—	147
4	Before	6·5	0·4	1·5	<10
	After	9·3	0·1	Plasma not jaundiced	155
27	Before	8·0	0·2	,,	90
	After	8·3	0·2	,,	120

Discussion

The results reported here indicate that the serum haptoglobins are usually low in patients with increased haemolysis. This confirms the earlier reports cited above and others which have appeared since this investigation began (Nyman, Gydell, and Nosslin, 1959; Lathem and Jensen, 1959; Brus and Lewis, 1959).

In a previous paper (Owen, Carew, Cowling, Hoban, and Smith, 1960b), the view was put forward that a low serum haptoglobin level associated with increased haemolysis is most likely due to the formation of a haemoglobin-haptoglobin complex in the plasma and to its subsequent removal from the circulation. The finding (Table V) of a negative correlation between haemoglobin destruction and serum haptoglobin supports this view, as does the negative correlation between serum bilirubin and haptoglobin levels observed in this investigation and in a previous study (Owen *et al.*, 1960b). Correlations between serum haptoglobin levels and faecal urobilinogen and endogenous blood carbon monoxide respectively (Nyman *et al.*, 1959) add additional support. The fact that the serum haptoglobins returned to normal in hereditary spherocytosis following splenectomy which resulted in complete clinical and haematological remission is further evidence that low serum haptoglobins in haemolytic states are related directly to the haemolytic process. Similar observations following splenectomy have been made by Chevallier and Wolpé (1945) and Brus and Lewis (1959).

Brus and Lewis (1959) demonstrated a correlation between haemoglobin destruction and serum haptoglobin levels; they found that in most of their cases the haptoglobins were absent when the haemoglobin turnover per day exceeded twice the normal rate. The data in Table V support this conclusion that the reduction in serum haptoglobins is related to the amount of haemoglobin destroyed. Thus Cases 1–4, patients with congenital haemolytic anaemia, in whom there was a significant increase in peripheral haemoglobin destruction, had low haptoglobin values. In the other cases listed (with the exception of Case 25) the calculated peripheral haemoglobin destruction rates were normal or only slightly increased and there was no decrease in serum haptoglobins; nevertheless most of these patients had a haemolytic state as evidenced by shortened red cell survival times. In these patients, all of whom had myelosclerosis, leukaemia, or lymphoma, the bone marrow was presumably unable to increase significantly the rate of erythropoiesis to compensate for the shortened red cell life span, as it could in the cases of congenital haemolytic disease. There are two possible explanations for the absence of a fall in serum haptoglobins in these cases: (1) The fact that the peripheral haemoglobin destruction rate was not significantly increased; (2) the underlying disorder may have caused some increase in haptoglobins (see below).

It appears that haptoglobins are reduced in both intravascular haemolysis, in which the haemoglobin is released into the circulation directly from disintegrating cells, and extravascular haemolysis in which red cells break down in the cells of the reticulo-endothelial system. Thus values were reduced in paroxysmal nocturnal haemoglobinuria (Case 11) in which haemolysis is

known to occur intravascularly, and in the cases of chemical haemolytic anaemia (Cases 12–14) in which the haemolysis was almost certainly occurring intravascularly. Low values were found in all cases of hereditary spherocytosis in which haemolysis has to date been regarded as occurring extravascularly. In the case of extravascular haemolysis some of the haemoglobin is presumably released from the reticulo-endothelial cells into the circulating plasma to form the haemoglobin-haptoglobin complex. Brus and Lewis (1959) in their extensive study also found that haptoglobin levels were reduced in both intravascular and extravascular haemolysis.

In some of the patients with leukaemia, myelosclerosis, and lymphomas the serum haptoglobins were increased. Raised serum haptoglobin levels occur commonly in other types of neoplasia and in disorders associated with tissue destruction or inflammation such as infections, collagen disease, trauma, and post-operative states (Chevallier and Wolpé, 1945; Jayle and Boussier, 1955; Allison and Blumberg, 1958; Nyman, 1959; Owen, Mackay, and Got, 1959; Brus and Lewis, 1959). The reason for this is not clear. It is probable that it is due to increased production of serum haptoglobins. Whatever the reason this is obviously a complicating factor in assessing the significance of serum haptoglobin levels in a particular case. Thus, an increased loss of haptoglobin (as a complex with haemoglobin) may be balanced by an increased production. Presumed examples have already been noted. A patient suffering from severe untreated pernicious anaemia and a fractured femur was found to have a normal haptoglobin level (Owen *et al.*, 1960b) though haptoglobin levels are usually considerably reduced in pernicious anaemia. Another patient with congenital haemolytic anaemia had normal haptoglobins during an upper respiratory tract infection though the serum haptoglobins were usually low (Chevallier and Wolpé, 1945). We have observed normal or raised serum haptoglobins in several patients with severe burns even though the patients had haemoglobinuria presumably due to haemolysis. Serum haptoglobins may also be increased by the administration of adrenocortical steroid hormones (Brus and Lewis, 1959). Case 24 showed a progressive rise in haptoglobins while under treatment with steroids; while this may have been in part due to a lessening in the rate of haemolysis, it probably was not entirely so, as signs of active haemolysis persisted during treatment.

From the point of view of the clinician the most important question is the value of serum haptoglobin determination in the diagnosis of haemolysis. Nosslin and Nyman (1958), in their preliminary communication, stated that "determination of the haptoglobin level is useful in the investigation of anaemia. A normal or raised level argues strongly against haemolysis; whereas low level in the absence of hepatic disease indicates haemolysis." It would certainly appear from other reports and from our observations that a low level, in the absence of hepatic disease, does indicate haemolysis. However, both our results and those of Brus and Lewis (1959) definitely indicate that a normal or raised level does not exclude haemolysis. Haemolysis without decrease in serum haptoglobins may occur (1) in the presence of malignancy or infection or adrenocortical steroid administration, and (2) when haemolysis is associated with impairment of bone marrow function with the result that the daily rate of haemoglobin destruction is not significantly increased; this is seen particularly in the leukaemias and malignant lymphomas.

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MULTIPLE PRIMARY CANCERS: PRIMARY MALIGNANT LYMPHOMAS AND CARCINOMAS OF THE INTESTINAL TRACT IN THE SAME PATIENT

BY

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Three cases of primary malignant lymphoma and primary mucus-secreting adenocarcinoma of the intestinal tract, occurring in the same patients, are described, and three similar cases reported in the literature are discussed. The carcinomas were present at the same time as the lymphomas, or developed after the lymphomas had been removed. The relationship between malignant lymphoma and carcinoma is uncertain, but there is some evidence to suggest a possible relationship between them. It is important not to assume that a second growth in the intestinal tract is always a recurrence or a metastasis from the original primary tumour.

Since Whipham first described a case of splenic leukaemia and carcinoma of the pancreas in 1878 and Billroth multiple primary cancers of the breast in 1879, multiple primary tumours, benign and malignant, have become a well-recognized and accepted phenomenon. The majority of multiple primary malignant tumours are carcinomas, and they tend to occur more commonly in the same organ, or in paired organs, than in different organs of the body (Slaughter, 1944). Most of the recorded cases are of carcinomas of the skin, colon, stomach, and breasts. If one excludes such diffuse lesions as leukaemia and malignant lymphoma, multiple primary sarcomas are uncommon, the best-recognized examples being the multiple neurogenic sarcomas of von Recklinghausen's disease and liposarcomas.

Carcinoma and sarcoma occurring in the same patient have been reported more frequently than multiple primary sarcomas, and most of these were carcinoma with leukaemia or malignant lymphoma. Moertel and Hagedorn (1957) described 120 such cases seen at the Mayo Clinic, and reviewed 194 similar cases collected from the literature. Weitzel (1958), reporting a further 29 cases seen at the Mallory Institute of Pathology, noted a special relationship between carcinoma and malignant disorders of plasma cells. Many recorded cases were carcinomas of the skin or gastro-intestinal tract, with lymphosarcoma or chronic lymphatic leukaemia. The lymphosarcomas were usually confined to lymph nodes at some distance from the carcinomas and were quite separate from them.

Primary malignant lymphoma and carcinoma, both situated in the intestinal tract of the same patient, is an unusual finding. Kreibig (1929) described the simultaneous occurrence of a lymphocytic lymphosarcoma of the terminal ileum in a 64-year-old man, with an adenocarcinoma of the caecum. The two tumours were quite separate, both had penetrated the muscle coats, and the lymphosarcoma had spread to the lymph nodes draining the terminal ileum. Cattell and Boehme (1947) described the simultaneous occurrence of a reticulum cell sarcoma of the caecum and an adenocarcinoma of the rectum in a woman aged 26 years. Warren (1959) described the simultaneous occurrence in a man of a primary malignant lymphoma of the rectosigmoid with an adenocarcinoma of the splenic flexure.

In a recent study of 38 cases of apparently primary malignant lymphomas of the small and large intestines, seen at St. Mark's, the Gordon, and Westminster Hospitals, London (Dawson, Cornes, and Morson, 1960), there were three cases of primary malignant lymphoma and carcinoma of the intestinal tract occurring in the same patients.

Case Reports

Case 1.—Mr. D. J., aged 63, first seen in November, 1946, complained of tenesmus, rectal bleeding, and slight weight loss. On examination a hard, friable,

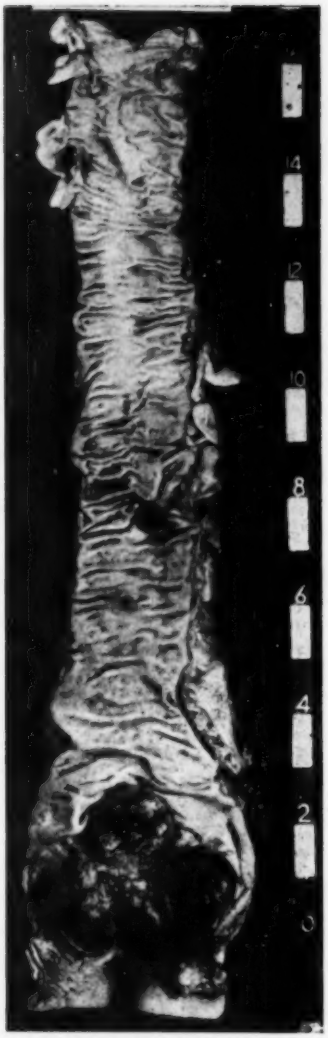

FIG. 1.—Case 1: Large lymphosarcoma lower third of rectum. Small carcinoma below lymphosarcoma, at the ano-rectal junction. Adenomatous polyp at recto-sigmoid junction.

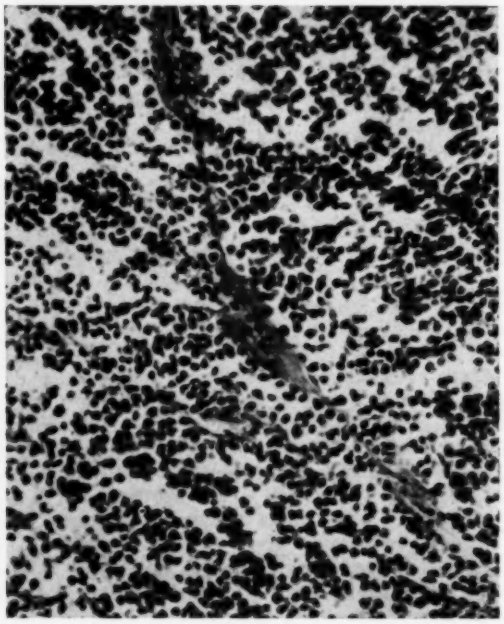

FIG. 2.—Case 1: Primary lymphocytic lymphosarcoma of rectum. Haematoxylin and eosin (×250).

ulcerating tumour was found in the rectum. There was no palpable lymphadenopathy, and a chest radiograph was normal. The haemoglobin was 16.6 g.%, and the white blood cells 8,500 per c.mm. An abdomino-perineal excision of the rectum was performed. At operation the liver and spleen appeared normal, and no enlarged lymph nodes were found. The patient made a quick recovery from the operation, and was then given a course of deep *x*-ray therapy. He died one year after the operation from uraemia, due to urinary obstruction caused by an enlarged prostate. There was no clinical evidence of recurrence of the tumours found in the rectum.

In the lower third of the excised rectum there was a large, protuberant growth, 8 cm. in diameter, almost completely encircling the bowel (Fig. 1). The overlying mucosa was ulcerated, and, on sectioning, the tumour was dark purple and fleshy in consistency. The tumour breached the muscle coats and infiltrated the surrounding perirectal fat. Just beneath this large tumour mass there was a second, smaller tumour, which on sectioning was seen to consist of numerous, small, cystic spaces, filled with mucus. A pedunculated polyp was present at the rectosigmoid junction, and the superior haemorrhoidal lymph nodes were slightly enlarged. Sections taken for histology showed a benign, adenomatous polyp of the rectosigmoid, a lymphosarcoma of the rectum, and a mucus-secreting adenocarcinoma of the rectum. The regional lymph nodes were free of growth. The large tumour mass was composed of small, round cells, with round, hyperchromatic nuclei, and scanty cytoplasm (Fig. 2). The muscle coats appeared to be eaten away by this tumour mass, and the lack of any surrounding fibrous reaction was striking. The appearances were

FIG. 3.—Case 1: Well-differentiated adenocarcinoma of rectum. Haematoxylin and eosin (×250).

those of a lymphocytic lymphosarcoma. The small tumour nodule was composed of large acini filled with mucus and lined by mucus-secreting columnar epithelium (Fig. 3). Although touching each other in the perirectal fat, the two tumours were quite discrete, and there was no invasion of one tumour by the other.

Case 2.—Mr. A. B., aged 52, first seen in April, 1946, complained of colicky abdominal pain, vomiting, and anorexia, for 10 days. On examination the abdomen was distended, but no other abnormality was found. The haemoglobin was 14.0 g.%, and the white blood cells 3,700 per c.mm. (84% neutrophils, 1% basophils, 1% eosinophils, 9% lymphocytes, and 5% monocytes). A chest radiograph was normal, and a plain radiograph of the abdomen showed the appearances of a small bowel obstruction somewhere in the lower ileum. A laparotomy was performed, and an area of thickening, 8 cm. long, was found in the terminal ileum. A diagnosis of regional ileitis was made, and the involved area was by-passed by a side-to-side ileo-ileal anastomosis. Two weeks later a second laparotomy was performed and the isolated loop of bowel was excised.

This operation specimen consisted of 21 cm. of ileum. In the area of growth the bowel wall was thickened, and the lumen grossly narrowed. On sectioning, the tumour was white and could be seen growing right through the muscle coats into the mesenteric fat. The overlying mucosa was intact. Sections taken for histology showed a lymphosarcoma. The histological appearances were similar to the lymphocytic lymphosarcoma of the first case (Fig. 4).

Following a course of deep *x*-ray therapy the patient remained in good health for 18 months, when he began to complain of colicky abdominal pain, vomiting, and constipation. A laparotomy was performed, and a stricture was found in the sigmoid colon. This stricture was resected, but the patient died four days later from a paralytic ileus. A necropsy was performed.

The operation specimen showed an adenocarcinoma of the sigmoid colon, completely encircling the lumen of the bowel. The tumour breached the muscle coats, and was infiltrating the surrounding pericolic fat. Microscopy showed a moderately well-differentiated adenocarcinoma penetrating the muscle coats and invading the local lymphatics (Fig. 5). At necropsy no tumour deposits of lymphosarcoma or carcinoma were found.

Case 3.—Mr. D. C., aged 52, first seen in November, 1955, complained of periodic attacks of abdominal pain after a gunshot wound of the abdomen in 1942. The pains became progressively more severe and generalized, and he developed anorexia and lost weight. On examination the abdomen was tender, there was no palpable abdominal mass, and no palpable superficial lymphadenopathy. The haemoglobin was 7.4 g.%, and the white blood cells 8,000 per c.mm., with a normal differential count. A chest radiograph was normal, and a barium meal with follow-through showed a stricture in the

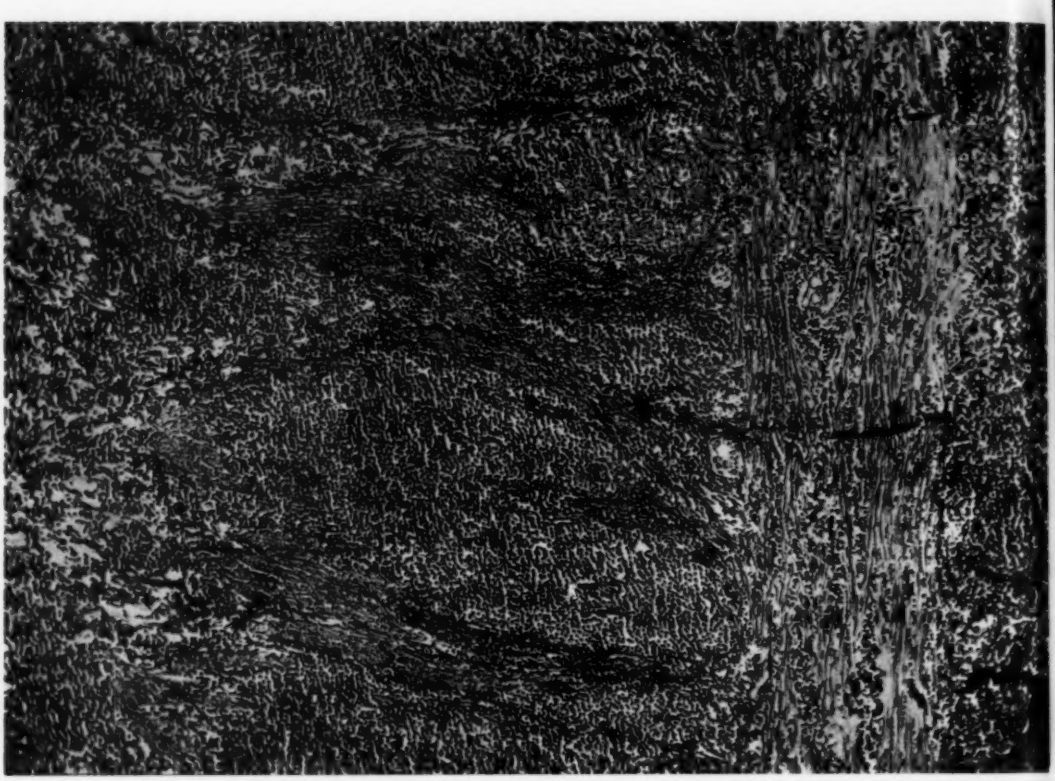

FIG. 4.—Case 2: Primary lymphocytic lymphosarcoma of terminal ileum. Note the punched-out appearance of the muscle coats and the absence of any fibrous reaction. Haematoxylin and eosin (×120).

ascending colon, multiple metallic fragments in the right iliac fossa, and stones in the gall-bladder. At laparotomy, in January, 1958, a large mass was found in the ascending colon, with the terminal ileum adherent to it in two places. The ileo-caecal lymph nodes were enlarged, but no other enlarged nodes were found at laparotomy, and the liver and spleen appeared normal. A right hemicolectomy was performed.

The operation specimen consisted of 25 cm. of terminal ileum and 21 cm. of caecum and ascending colon. The tumour mass in the ascending colon extended along the bowel for a distance of 13 cm. On sectioning, the tumour could be seen growing right through the bowel wall into the pericolic fat and mesentery, and at two sites the tumour was infiltrating the adjacent terminal ileum. Sections from the tumour mass showed a reticulum cell sarcoma. The cells were large and pleomorphic, with abundant pale-staining cytoplasm and large oval nuclei (Fig. 6). Silver stains demonstrated an abundant reticulin network amongst the tumour cells. The regional lymph nodes were free of growth.

Following a course of deep *x*-ray therapy the patient remained in good health for nine months, when he began to complain of passing blood and mucus per rectum. Sigmoidoscopy revealed multiple tumours and several small polyps in the rectum and sigmoid colon. An abdomino-perineal excision of the rectum, with removal of the sigmoid and part of the descending colon, was performed, and three months later the patient was alive and well.

The operation specimen was 30 cm. long. Numerous, small, sessile polyps were present throughout the rectum and sigmoid colon. Two large pedunculated polyps and three obviously malignant growths were also present (Fig. 7). The malignant growths were situated at 5, 13, and 20 cm. respectively from the ano-rectal junction. Microscopy showed all the tumours to be mucus-secreting adenocarcinomas, the two lowermost growths arising in villous papillomas. In addition, one of the pedunculated polyps had undergone a malignant change, and the stalk and adjacent submucosa were invaded by growth. The tumour at 5 cm. had invaded the submucosa

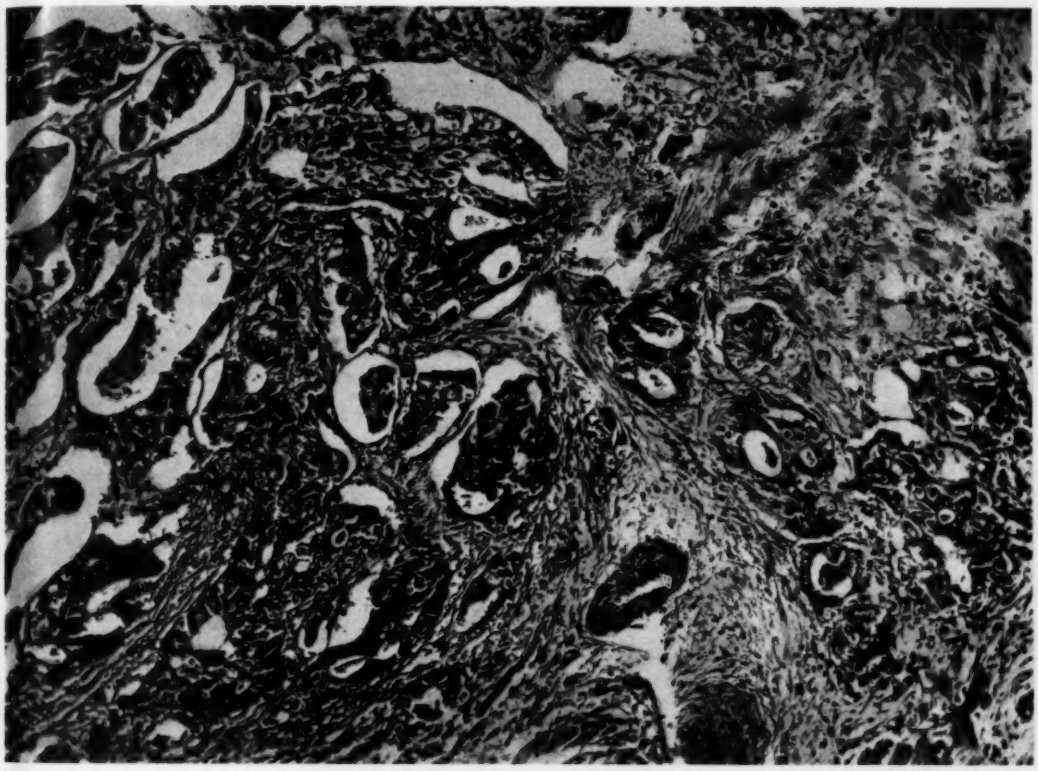

FIG. 5

FIG. 5.—Case 2: Moderately well-differentiated adenocarcinoma of sigmoid colon. Haematoxylin and eosin (×120).

FIG. 6.—Case 3: Primary reticulum cell sarcoma of ascending colon. Haematoxylin and eosin (×650).

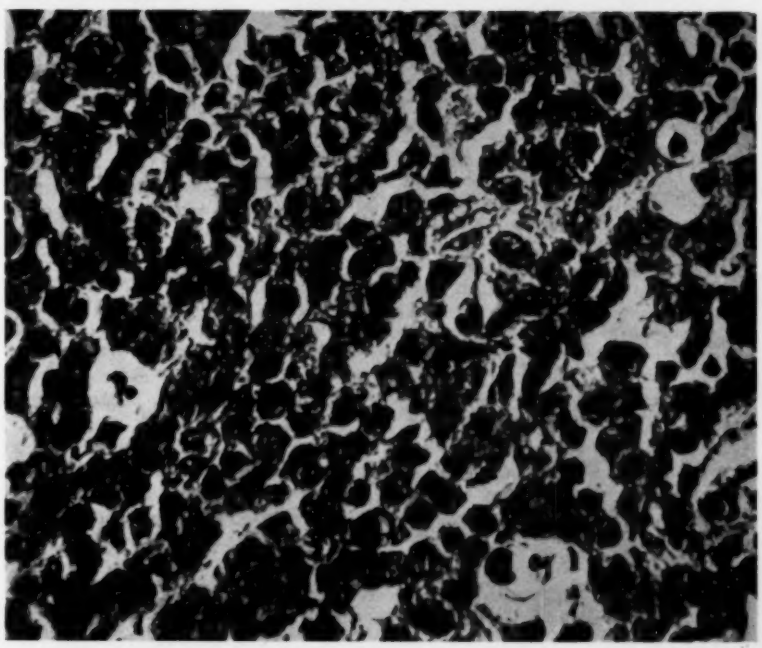

FIG. 6

FIG. 7.—Case 3: Multiple carcinomas of rectum and sigmoid colon.

only, but those at 13 and 20 cm. had breached the muscle coats. The neighbouring lymph nodes were invaded by adenocarcinoma, but the more distant lymph nodes were free of growth.

Discussion

Primary malignant lymphomas of the intestinal tract are frequently multiple, and in the series of 38 cases reported by Dawson *et al.* (1960) there were eight cases of this type. The occurrence of malignant lymphoma and adenocarcinoma of the intestinal tract in the same patient, on three occasions, is, however, a most unusual and remarkable finding. Clinically the lymphomas appeared to be primary in the intestines, because there was no palpable lymphadenopathy, chest radiographs showed no obvious mediastinal glandular enlargement, and repeated blood counts excluded the possibility of leukaemia. At laparotomy, the liver and spleen appeared normal in all cases, and the only lymph nodes obviously involved were those in the immediate neighbourhood of the bowel tumours. Post-operatively none of these three cases developed a generalized lymphoma or leukaemia, and in the second case a necropsy showed no tumour anywhere else in the body. In all cases the lymphomas penetrated the muscle coats and infiltrated the surrounding fat, a feature regarded by Stout (1955) as an important feature of primary malignant lymphomas in the gut. The carcinomas were all mucus-secreting, and were readily distinguished from the malignant lymphomas.

In the cases described by Cattell and Boehme (1947) and Warren (1959), the malignant lymphomas and carcinomas occurred in cases of long-standing ulcerative colitis. Carcinoma is a well-recognized complication of chronic ulcerative colitis (Dawson and Pryse-Davies, 1959), but lymphosarcoma complicating this disease has been less frequently described (Cornes, Smith, and Southwood, 1960).

No significant relationship between malignant lymphoma and carcinoma has yet been established. Moertel and Hagedorn (1957), describing 68 cases seen at the Mayo Clinic, concluded that the presence of a malignant lymphoma did not seem to predispose to the development of any other specific type of primary malignancy, and that the incidence of another primary malignant lesion in patients with a malignant lymphoma only slightly exceeded that in any other segment of the general population of similar age. Tissue culture studies and animal inoculation experiments offer some suggestion of a relationship between malignant lymphoma and carcinoma. In tissue cultures, the lymphocyte is the only cell to be attracted to areas of mitosis, is the only motile cell to attach itself to other cells for long periods of time, easily enters and leaves malignant cells, and can wander freely about inside their cytoplasm (Pulvertaft, 1959). Salaman (1959), using cell-free filtrates of mouse leukaemic tissue injected subcutaneously into newborn mice, produced 40 tumours in 15 out of 23 inoculated mice which survived for three months or more. Twenty-six of these were carcinomas, and no case of leukaemia was found. It is interesting to note that of the three present cases, and the three cases reported

in the literature, there was no case of carcinoma preceding the lymphoma. In four cases the tumours were synchronous, and in two cases metachronous, the carcinomas following the lymphomas within 18 months.

The finding of a second primary cancer in the intestinal tract is of considerable clinical importance, since the results of surgical removal of primary malignant lymphomas of the gut are most encouraging, and the prognosis for carcinomas of the colon and rectum, if removed early enough, is equally encouraging. It is therefore most important to have an adequate clinical follow-up in all cases, and not to be too ready to assume that a second growth in the intestinal tract is a recurrence or a metastasis from the original primary tumour.

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THE WEIGHTS OF KIDNEYS IN LATE INTRA-UTERINE LIFE AND CHILDHOOD

BY

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Statistical tables including 50 and 95 percentile ranges are presented of the combined kidney weights compared with body weight, crown-rump length, and gestation age of foetuses and newborn children, and also a table of kidney weights related to post-natal age. The importance of relating kidney weight to more than one general body criterion in the perinatal period is illustrated.

It is difficult enough to estimate normality in living newborns and infants, but when we come to estimate the normal weights of organs from infants that have died in the perinatal period, largely from diseases not known to us, the difficulties are much greater. The early work in this field and the problems involved were well reviewed by Cruickshank and Miller (1924a and b) and Browne (1924).

The weights of different organs for the first few months of intra-uterine life were worked out by Clatworthy and Anderson in 1944. The present standard weights for older foetuses were established by Potter and Adair (1949) and Potter (1952). They grouped cases in such a way that crown-rump length and gestation age and body weight were brought together in the same groups. Their method, which follows that used by many of the earlier workers in this field (Giese, 1898; Ylppö, 1919; Feldman, 1920; Cruickshank and Miller, 1924b; and Browne, 1924), is excellent for elucidating the general principles of differential growth of organs, but does not supply the particular information sometimes required by the pathologist.

For later childhood, the best established normal weights are those of Coppoletta and Wolbach (1933). Since then the general weight and height of children has increased both in Europe and America (Provis and Ellis, 1955; Stein, 1955; Thomson, 1956; *Lancet*, 1956). Keddie (1956) suggests that during the past 40 years there has been an increase of approximately 7½ lb. in weight and 3 in. in height in 9-year-old children.

The present paper is part of a general study being carried out in Sheffield to establish the normal weights of different organs of infants and children at the present time in England.

Material and Methods

The kidneys used in this study came from the records of 2,500 necropsies carried out by the Department of Pathology at the Children's Hospital, Sheffield, from 1950 to 1960. Throughout this time the necropsies were carried out with the definite view of assessing the normal weights of organs. Kidneys were fixed uncut, while still attached to their blood vessels. After fixation the kidneys were separated from the ureters and blood vessels, and as much of the perirenal fat as possible was removed, but the capsule was not stripped. The kidneys were weighed together on a regularly tested Avery scale correct to the nearest half-gram.

All material was surveyed, and all cases showing abnormality of the renal tract, multiple congenital deformities, gross post-mortem changes, or histological abnormality of either kidney were eliminated. This left approximately 1,300 cases.

The combined weights of the kidneys were charted separately against crown-rump length, body weight (in grams), gestation age (estimated from the first day of the mother's last menstrual period) and post-natal age irrespective of gestation age at birth. These measurements were not complete in all cases, thus the number of cases used in the different correlations was not identical. Means, standard deviations, standard error of means based on the formula $SE = \sqrt{\frac{\sigma}{n}}$, and 50 and 95 percentile ranges were calculated.

Results and Discussion

A great variation in the range of weights of kidney was found for all criteria, but no greater than that found in the more recent studies on adult kidneys (Wald, 1937). The findings are given

TABLE I

KIDNEY WEIGHT RELATED TO CROWN-RUMP LENGTH

Crown-rump Length (cm.)	Mean (g.)	S.E.	50% Range	95% Range	Potter and Adair (1949)
22	7·0	0·65	4·5– 8·0	2·0–11·0	
23	8·5	0·65	5·5– 9·5	3·0–12·0	
24	9·5	0·55	7·0–11·5	4·0–15·0	9·7
25	11·0	0·55	8·0–13·0	5·0–16·0	
26	12·5	0·55	9·5–15·0	5·5–17·5	
27	14·0	0·3	11·0–17·0	6·5–19·5	
28	15·5	0·3	12·5–19·0	7·0–21·5	13·6
29	17·0	0·3	14·0–20·5	7·5–23·5	
30	18·5	1·4	16·0–22·5	8·0–26·0	
31	20·0	1·12	17·0–24·5	8·5–28·0	18·3
32	21·5	0·85	18·5–26·0	9·0–31·0	
33	23·0	0·88	20·0–28·5	10·0–32·5	21·1
34	25·0	0·92	21·5–30·5	10·0–35·0	
35	26·5	0·94	23·0–32·0	11·0–38·0	23·6
36	28·5	0·94	24·0–34·0	12·0–41·0	26·6
37	30·5	1·37	26·5–36·0	13·0–45·0	29·3
38	33·0	1·37	27·0–38·0	14·0–48·0	
39	36·0	1·48	28·0–40·5	14·5–52·5	32·2
40	39·0	1·48	30·0–43·0	15·5–56·5	
41	41·5	1·48	32·5–46·0	16·5–61·0	
42	45·5	1·48	36·0–50·0	18·0–66·0	
43	50·0	1·48	40·0–53·5	19·0–71·0	
44	53·5	3·75	43·0–58·0	21·0–75·5	
45	57·0	3·75	48·0–61·5	22·5–81·5	
46	61·5	3·75	51·0–65·5	24·0–88·0	
47	65·0	3·75	54·0–69·5	26·0–93·0	
48	69·0	3·75	57·0–73·5	28·0–99·0	
49	73·0	3·75	60·5–77·5	30·0–105·0	
50	77·0	3·75	64·0–82·0	32·5–113·0	

TABLE II

KIDNEY WEIGHT RELATED TO TOTAL BODY WEIGHT

Body Weight (g.)	Mean	S.E.	50% Range	95% Range	Potter and Adair (1949)
1,400	15·5	1·03	14·0–20·5	9·5–25·5	
1,500	17·0	1·03	14·5–21·0	10·0–26·0	
1,600	18·0	1·03	15·5–21·5	10·5–27·0	
1,700	19·0	0·83	16·0–22·0	11·0–27·5	
1,800	19·8	0·83	16·5–23·0	11·5–28·0	
1,900	20·5	0·83	17·5–23·5	12·0–29·0	
2,000	21·0	0·83	18·5–24·5	13·0–30·0	18·3
2,100	22·0	0·83	19·0–25·0	13·5–31·0	
2,200	23·0	1·24	19·5–25·5	14·0–32·0	
2,300	23·5	1·24	20·5–26·5	14·0–33·0	
2,400	24·0	1·24	21·0–27·0	14·5–34·5	
2,500	25·0	0·81	22·0–28·0	15·0–35·0	21·1
2,600	25·5	0·81	22·5–29·0	15·0–35·5	
2,700	26·0	0·81	22·5–30·0	15·0–37·0	
2,800	27·0	0·98	23·0–31·0	15·0–38·0	
2,900	27·5	0·98	23·0–32·0	15·5–39·0	
3,000	28·5	0·98	23·5–33·0	15·5–40·0	23·6
3,100	29·0	0·98	23·5–34·0	15·5–41·5	
3,200	29·5	1·46	24·0–34·5	16·0–42·5	
3,300	30·0	1·46	24·5–35·5	16·5–43·5	
3,400	31·0	1·46	25·0–36·0	17·0–45·0	
3,500	31·5	1·27	26·0–36·5	17·5–46·0	26·6
3,600	32·5	1·27	26·5–37·0	18·0–47·0	
3,700	33·0	1·27	27·5–38·0	18·0–48·5	
3,800	33·5	1·27	28·0–38·5	18·5–49·0	
3,900	34·5	1·27	29·0–39·0	19·0–50·5	29·3
4,000	35·0	2·00	30·0–40·5	20·0–52·0	

in Tables I to IV. Comparative figures, where available, are included. Kidney weights graphed against post-natal age are also presented in Fig. 1.

It is thought essential that the weights be presented separately, against body weight, gestation age, and length, and not combined, as Potter and Adair found convenient, due to the differences found in the variation from "normal" under different criteria. This is illustrated in Fig. 2, where the scatter diagrams of the kidney weights against crown-rump length and body weight are shown. In the chart using the total weight of the child, three cases, A, B, and C, are well outside the 95 percentile ranges. The same cases in the crown-rump length chart are not only well within the 95 percentiles, but close together. Thus, against body weight, these body weights are grossly abnormal, whereas against crown-rump length they are quite normal.

TABLE III

KIDNEY WEIGHT RELATED TO GESTATION AGE

Gestation Age (wk.)	Mean	S.E.	50% Range	95% Range	Potter (1952)
28	11·0	2·49	9·5–13·5	4·5–19·5	9·7
29	12·0	2·29	10·0–14·5	5·0–21·0	
30	13·0	2·20	11·0–16·0	5·5–22·5	
31	14·5	2·16	12·0–17·0	6·0–24·0	13·6
32	16·0	2·75	13·0–18·5	6·5–25·5	
33	17·0	3·33	14·0–19·5	7·0–26·5	
34	18·0	3·97	15·0–20·5	7·5–27·5	
35	19·5	4·61	16·5–22·0	8·5–30·0	18·3
36	20·5	3·50	17·0–23·0	9·0–31·5	
37	21·5	3·50	18·0–24·5	9·5–33·5	21·1
38	23·0	3·50	19·0–26·0	10·0–35·5	
39	24·0	2·38	20·0–27·5	11·0–37·5	23·6
40	25·5	2·87	21·0–29·5	11·5–39·5	26·6
41	27·0	3·36	21·5–31·0	12·5–41·0	32·2
42	28·0	4·41	22·5–32·5	13·0–43·0	
43	29·5	5·46	23·5–34·5	14·0–45·0	
44	30·5	6·12	24·5–36·0	15·0–47·0	
45	31·5	6·79	26·0–38·0	16·0–49·0	

TABLE IV

KIDNEY WEIGHT RELATED TO POST-NATAL AGE

Age	Mean	S.E.	50% Range	95% Range	Coppolletta and Wolbach (1933)	Tkatschenko (1900)
Birth	26	0·76	22– 34	13– 44	27	22–24
1 mth.	32	0·98	26– 38	16– 50	30	22·8–24·8
2 ,,	36	1·13	29– 42	18– 54	37	
3 ,,	39	1·43	33– 46	21– 60	39	32–36
4 ,,	44	1·73	36– 50	24– 65	43	
5 ,,	48	1·73	40– 54	27– 70	50	45·2–47·2
6 ,,	51	1·73	43– 58	30– 76	51	
7 ,,	56	2·04	46– 63	32– 82	60	58–60
8 ,,	60	2·04	50– 68	35– 88	61	
9 ,,	62	2·04	54– 72	38– 95	61	58–60
10 ,,	67	2·04	58– 78	40–100	63	
11 ,,	69	2·04	63– 85	43–109	67	
12 ,,	78	2·04	68– 92	48–118	71	72–74
2 yrs.	88	2·74	74–103	54–130	93	
3 ,,	100	5·2	81–114	62–142	97	95–100
4 ,,	110	5·2	92–126	72–156	114	
5 ,,	121	5·2	100–136	81–169	129	110–112
6 ,,	134	6·4	110–150	90–182	135	
7 ,,	144	6·4	120–160	101–194	139	
8 ,,	156	6·4	131–173	111–207	149	
9 ,,	166	6·4	142–185	120–220	165	148–152
10 ,,	178	6·4	153–196	130–230	187	

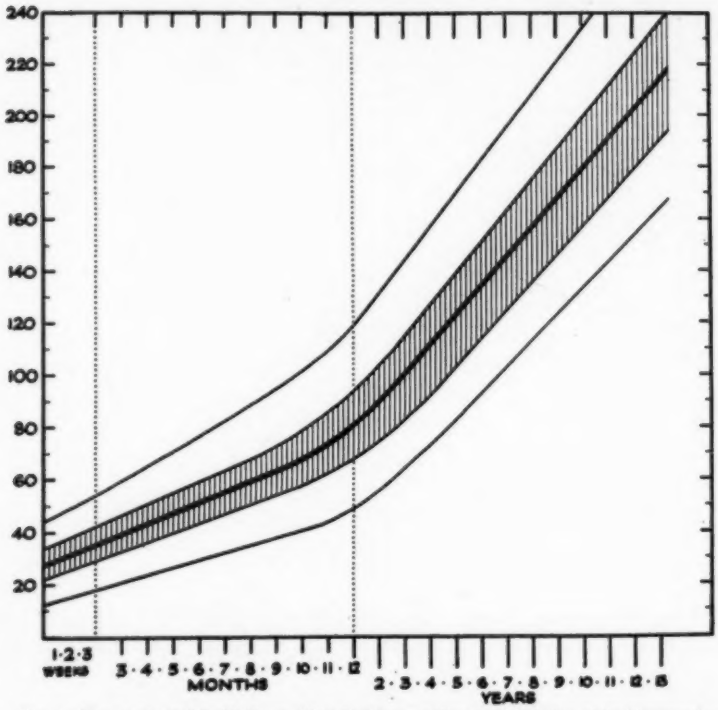

FIG. 1.—The mean combined kidney weights, the 50 percentile range (shaded), and the 95 percentiles (unshaded), the kidney weight being related to post-natal age, from 804 pairs of kidneys.

The method of estimation of the normal range used is that of percentiles rather than standard deviation. This problem was discussed well by Boyd (1935), who concludes that percentile ranges are the most useful as was originally stressed by Sir Francis Galton in 1885.

Our findings for post-natal kidney weight show slightly lower values than those of Coppoletta and Wolbach (1933). This is surprising because our method did not permit the kidneys to be squeezed and emptied of blood before weighing. Also, the general body weight and height of these children are probably higher than those of children used in the Boston series of 25 years ago.

The foetal kidney weights given by Cruickshank and Miller (1924b) and Browne (1924), from this country, compare very closely with our figures, suggesting that foetal kidney weights have not altered over the last 25 years. In general our weights show a higher range than those of Potter and Adair (1949) and Potter (1952), but their presentation is not strictly comparable to the present study as they produced common body weight, age, and body length grouping. We have shown that quite different results can be obtained when the kidney weight is related to the total body weight than when related to crown-rump length, and the same applies to gestation age.

When attempts are made to elucidate intra-uterine disease, estimates should be made against the different general body measurements separately.

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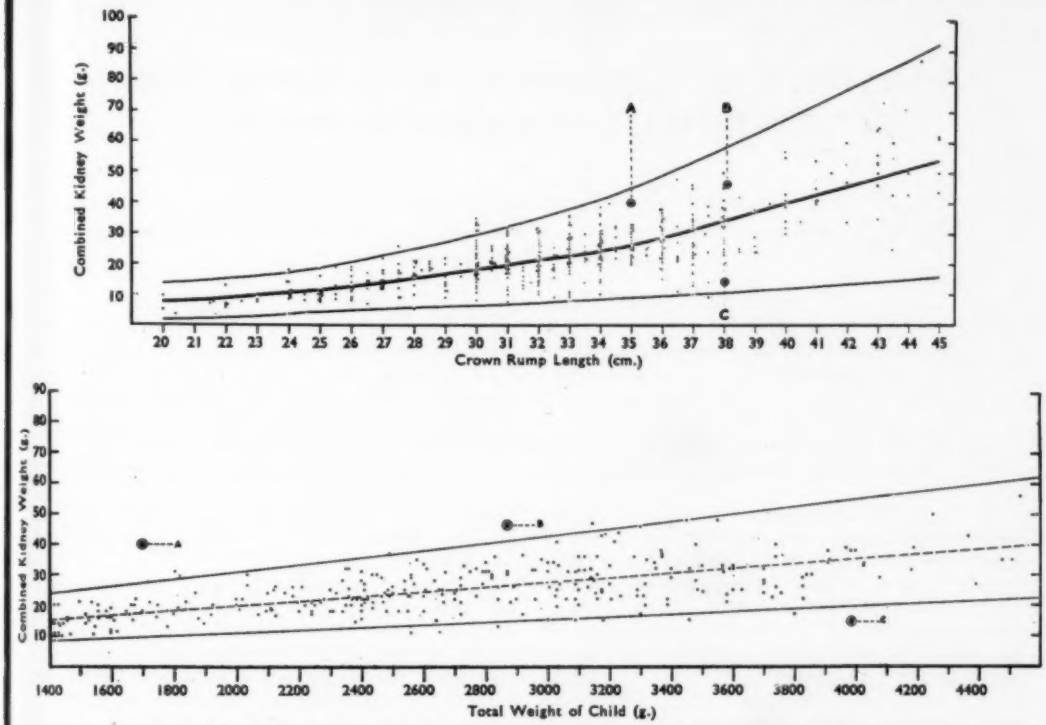

FIG. 2.—Scatter diagrams of the combined kidney weights with the means and 95 percentile lines against crown-rump length and total weight of the child in grams. The points marked A, B, and C represent the same kidneys as they fall in the different graphs.

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PARATHYROID ADENOMA WITH MISLEADING PHOSPHATE EXCRETION TESTS

BY

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A patient with primary hyperparathyroidism is described in whom, pre-operatively, the renal tubular handling of phosphate was within the limits accepted by recent writers as normal. The tests they advocate were, therefore, shown to be unreliable in this disease. The hypercalcaemia of primary hyperparathyroidism is one type of hypercalcaemia that is apparently not reduced to normal after the administration of an adequate dosage of cortisone. In our patient, such a " cortisone-fast " finding is documented, and was a valuable aid in the decision to operate.

In the earlier descriptions of primary hyperparathyroidism most of the patients were suffering from symptomatic bone disease, the osteitis fibrosa cystica of von Recklinghausen (Norris, 1947). To-day most examples of hyperparathyroidism come to light in the investigation of patients with calcification in the urinary tract. In the literature from 1948 to 1954, of 236 patients with hyperparathyroidism, 178 (75%) had renal tract disorders, chiefly stone or nephrocalcinosis (Albright and Reifenstein, 1948; Black, 1953; Hellström, 1954), and by 1955 Black states that only about 5% of patients with hyperparathyroidism have "osseous complications." On the other hand, of several hundred patients with urinary calculi examined in that era, just under 7% were found to have hyperparathyroidism (Albright and Reifenstein, 1948; Beard and Goodyear, 1950).

In such patients the diagnosis hinges on the finding or exclusion of the biochemical criteria of hyperparathyroidism. Of these, hypercalcaemia is a *sine qua non*, although it is not pathognomonic. If it be accompanied by a low fasting plasma-inorganic-phosphorus level then hyperparathyroidism is likely, but unfortunately parathyroid overactivity cannot be excluded if the plasma phosphorus is normal, for Goldman, Gordan, and Chambers (1957) have shown that a high calcium and normal phosphorus can occur in hyperparathyroidism even in the absence of renal failure.

In the continued lack of a diagnostic hormone assay, various additional chemical investigations have recently been proposed as aids to the diagnosis, especially of the milder forms of the disease. These tests have mainly been focused on phosphaturia.

Change in inorganic-phosphate reabsorption by the renal tubules was suggested by Crawford, Osborne, Talbot, Terry, and Morrill (1950) as an index of parathyroid function, and a striking increase in renal phosphate reabsorption following parathyroidectomy in man was described by Sirota (1953). Other workers (Schaaf and Kyle, 1954; Nordin and Fraser, 1956; Bogdonoff, Woods, White, and Engel, 1956; Goldman *et al.*, 1957; Kyle, Schaaf, and Canary, 1958) have now described a variety of tests for renal handling of phosphate, with the suggestion that these are of real significance in the positive identification or exclusion of primary hyperparathyroidism.

In the patient to be described, with a proved parathyroid adenoma, the pre-operative renal handling of phosphate, as measured by several of the published tests (Bogdonoff *et al.*, 1956; Goldman *et al.*, 1957; Kyle *et al.*, 1958; Nordin, 1958), indicated normal parathyroid function. Fortunately for the patient the implications of those results were not accepted as a contra-indication to surgical exploration.

Case Report

A 57-year-old widow was admitted in January, 1958, for investigation of hypercalcaemia. In October, 1956, and again in March, 1957, she had brief episodes of left renal colic. Other complaints at these times were long-standing backache, constipation, dysphagia, and dyspepsia, and recently frequency of micturition, with nocturia. Urine analysis was

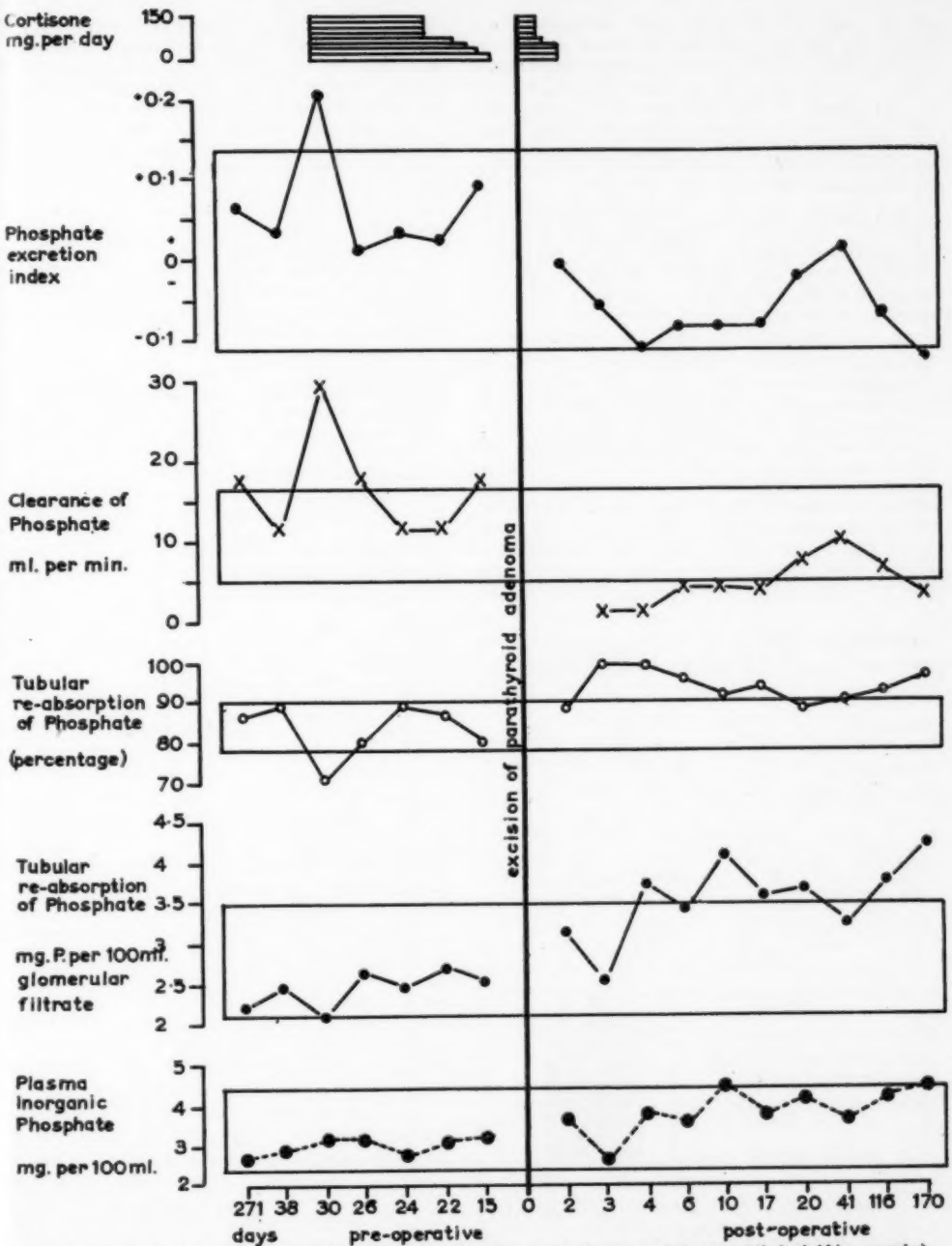

FIG. 1.—Results of renal tubular handling of phosphate expressed in four ways. These are within normal limits (within rectangles) before parathyroidectomy, except for one result. Note that this time scale is not linear.

always negative, no renal tract abnormality was shown by radiography, and she could concentrate to S.G. 1027. In 1957, plasma calcium values ranged between 11.8 and 13.0 mg. per 100 ml., but the corresponding plasma inorganic phosphorus values (probably not fasting) were within the normal range for this hospital, between 2.4 and 2.9 mg. per 100 ml. On a low calcium diet, successive 24-hour urinary calcium values were 350, 416, and 520 mg.

Past Health.—She had a severe Plummer–Vinson syndrome about 1938. A gastric ulcer of the lesser curvature was shown in 1948. For 12 months in 1951 and 1952, from 50,000 to 150,000 international units of ergocalciferol was given daily for lupus vulgaris of the cheek.

Present Admission.—In January, 1958, she looked plump and fit, but had tenderness of the left loin and several patches of active lupus vulgaris on one cheek. Band calcification was seen in both corneae.

The chemical findings included persistent hypercalcaemia and normal plasma phosphorus levels. The blood urea and plasma electrolytes were normal and there was no proteinuria. Endogenous creatinine clearances ranged between 89 and 94 ml. per minute. The tubular reabsorption of phosphate (T.R.P.) figures were also within the normal limits of published criteria (vide supra) (Figs. 1 and 2), the 24-hour urinary output of phosphorus being throughout in the range 714–850 mg., i.e., the patient was not on a low phosphate intake. Blood was always withdrawn at the same time each morning, with the patient in the fasting state to avoid post-prandial and diurnal variations in blood levels (Stanbury, 1958).

An exhaustive search was made for neoplasm. Oesophagoscopy showed a simple "web" stricture; dilatation of this relieved dysphagia. A chest radiograph, a barium meal, and tests for faecal occult blood were negative. Marrow from the sternum and ilium showed no infiltrate; she had a mild hypochromic microcytic anaemia (Hb 10.9 g. per 100 ml.). An extensive, but negative, survey was made of the bones, particularly for the subperiosteal bone resorption and other changes of hyperparathyroidism (Pugh, 1951).

Hyperparathyroidism seemed the probable cause of the hypercalcaemia, but because no abnormality in phosphate metabolism could be demonstrated, we undertook the cortisone test advocated by Dent (1956). Oral cortisone acetate was given continuously for 18 days, 150 mg. daily for the first 10 days, after which the dose was tapered off over eight days. As no significant change occurred in the serum calcium level (Fig. 1), we recommended operation.

Operation.—On February 15, 1958, a 2.25 g. right upper parathyroid adenoma was removed, microscopically of chief-cell type.

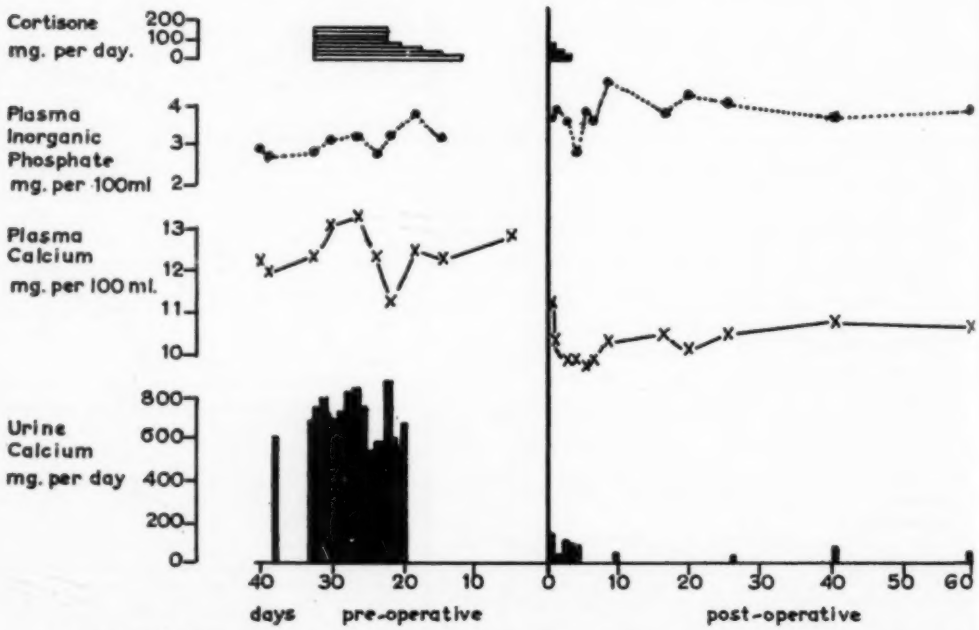

FIG. 2.—Results of the "cortisone" test and of excision of the parathyroid adenoma. Cortisone did not affect significantly the level either of the plasma or of the urinary calcium.

Progress.—Twenty-four hours later, the plasma calcium had fallen to 10.1 mg. per 100 ml. and thereafter fell further to 9.4 mg. per 100 ml., but soon stabilized (Fig. 1). Tetany did not occur. The plasma phosphorus values rose equally rapidly, to high normal levels; there were corresponding changes in the various calculated indices of renal tubular handling of inorganic phosphate. Indeed, some of these indices remained for months at levels indicative of hypoparathyroidism (Fig. 2), thus confirming the ablation of a functional tumour. The 24-hour urinary output of calcium and of phosphorus also fell at once post-operatively; the phosphorus output attained previous levels after about six weeks, and the urinary calcium remained at under 100 mg. per 24 hours for six months.

Symptomatically the patient noticed an early improvement in her sense of general well-being and also complete freedom from her backache, left flank pain, and chronic dyspepsia; she gained 9 lb. in eight weeks. This sense of well-being has been maintained. Six months later the corneal calcification had lessened but left some fine scarring.

Comment

Other than hyperparathyroidism, there are many conditions associated with hypercalcaemia (Thomas, Connor, and Morgan, 1958). These include myeloma and a wide variety of other malignant diseases, sarcoidosis, the milk-alkali syndrome, hypervitaminosis D, the idiopathic hypercalcaemias of infancy, hyperthyroidism (Koenig, Scholz, and Salassa, 1957), adrenal insufficiency (Leeksma, De Graeff, and De Cock, 1957), and extensive skeletal immobilization even of normal people, but especially of those with Paget's disease (Stein, Stein, and Beller, 1955). Thorough physical examination and appropriate investigations will identify some of these states, though neoplastic involvement of bone may be present without radiological evidence (Plimpton and Gellhorn, 1956). Hypercalcaemia itself may cause indeterminate symptoms indistinguishable from those of hyperparathyroidism, such as anorexia and constipation (Albright and Reifenstein, 1948), and this simulation is even more marked in the rare case where hypercalcaemia is accompanied by hypophosphataemia (Thomas *et al.*, 1959). Hypercalciuria is of no differential value, as it occurs with any rise in plasma calcium and also is now known to be common among patients with renal calculi who have neither hypercalcaemia nor hyperparathyroidism (Pyrah, 1958); vitamin D in high dosage too can cause hypercalciuria without hypercalcaemia (Howard, 1953).

The importance and difficulty of deciding the cause of hypercalcaemia are shown in the efforts to evolve more informative tests of the action of the parathyroids on the kidney, but the pity is that undue reliance on these diagnostic refinements has already led to useless surgical explorations both published (McGeown and Montgomery, 1956; Beare and Knudsen, 1956) and unpublished. There was a failure to appreciate the influence of such factors as diet, diurnal variation, the absolute level of plasma phosphorus (Stanbury, 1958) and depressed renal function (Goldman and Bassett, 1954) on these newer measurements of hyperphosphaturia, especially on the phosphate clearance and the percentage tubular reabsorption of phosphate. Attempts to overcome the effects of diverse plasma phosphorus levels were made in the phosphate excretion index of Nordin and Fraser (1956), and in the absolute expression "mg. of phosphate reabsorbed per 100 ml. glomerular filtrate" (Thomas *et al.*, 1958).

In reviewing the results of such tests on 118 patients with renal calculi, McGeown (1957) has now reported seven examples of tests falsely indicating hyperparathyroidism in patients with persistently normal serum and urinary calcium values. More recently, "false positive" results with a low percentage tubular reabsorption of phosphate have also been shown in seven hypercalcaemic patients without primary hyperparathyroidism (Thomas *et al.*, 1958).

In our patient renal function was normal, as judged by endogenous creatinine clearance and concentration tests, the pre-operative renal phosphate handling was within normal limits by published standards (Fig. 2) and yet a functioning parathyroid adenoma was present. Removal of this adenoma was rapidly followed by significant enhancement of tubular phosphate reabsorption, as shown by a reduction in urinary phosphorus excretion and an increase in plasma phosphorus. It was only in retrospect, by comparison of the post-operative and the apparently normal pre-operative values, that the degree of phosphaturia before the operation could be recognized as being relatively high. Thus this patient might have been denied the benefits of operation had the finding of "normal" phosphate handling been accepted as excluding hyperparathyroidism (Goldman *et al.*, 1957; Kyle *et al.*, 1958; Nordin, 1958).

Our patient is not unique in this respect, since McGeown (1957) has already mentioned three patients with proved hyperparathyroid states in whom the plasma phosphorus levels in the blood were normal and the percentage of phosphate reabsorption by the tubules was also normal.

The hypercalcaemia of hyperparathyroidism is said by Dent (1956) and Connor, Hopkins, Thomas, Carey, and Howard (1956) to show a specific resistance to lowering by the administration of cortisone, and although this has not yet been widely confirmed (Thomas *et al.*, 1959) no exceptions are known to us. It was mainly on the findings with this test, taken with the clinical background, that we were encouraged to make a firm diagnosis of hyperparathyroidism. Apart from the benefit to the patient, this result encourages further evaluation of the response to cortisone in the analytical diagnosis of hypercalcaemia.

We thank Professor A. C. Lendrum, Professor I. G. W. Hill, and Dr. K. G. Lowe for continuous encouragement, Miss M. R. Haslock, B.Sc., for the many technical estimations, Dr. A. A. Douglas for the slit lamp observations, and Mr. J. Mowat for the oesophagoscopy.

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THE RELATION OF THYROID AUTO-IMMUNITY TO ROUND-CELLED INFILTRATION OF THE THYROID GLAND

BY

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The relationship between thyroid auto-immunity and the presence of lymphocytes in the thyroid gland has been analysed in patients with Graves' disease, toxic adenoma, and non-toxic nodular goitre. In all these conditions circulating antibody to thyroglobulin was significantly associated with lymphocytic infiltration. No similar correlation was established in the case of the complement-fixing antibody.

Complement-fixing antibody was significantly commoner in Graves' disease (diffuse thyrotoxic goitre) than in toxic adenoma and non-toxic nodular goitre. Circulating thyroglobulin antibody probably originates from lymphoid tissue within and around the thyroid gland itself.

Overactivity of pituitary thyrotropin may favour immunization to the intracellular (complement-fixing) antigen.

Circulating auto-antibodies to thyroid constituents, which are usually present in Hashimoto's thyroiditis and non-goitrous myxoedema, can also be demonstrated in a proportion of cases of Graves' disease and non-toxic adenomatous goitre (see recent reviews by Roitt and Doniach, 1960; and Smart and Owen, 1960). So far as we are aware, no systematic study has been made of the relationship in the latter conditions between serological reaction and the histological appearance of the thyroid gland. Roitt and Doniach (1958) stated that localized lymphoid infiltration was found at operation in the majority of their patients with thyrotoxicosis who gave positive antibody tests and in some who did not. In nine patients without clinical thyroid disorder whose sera had given "false positive" complement-fixation reactions with thyroid extract, Goudie, Anderson, and Gray (1959) were able to examine the gland at necropsy and noted the presence in eight of these of lymphocytic foci and Askanazy-cell change; similar appearances were also found in nine subjects who had given negative complement-fixation reactions. On the basis of this partial correlation, it was suggested that the histological abnormality might be due to the action of auto-antibody on thyroid tissue. The value of the study, however, was lessened by being limited to the less specific of the two identified auto-immune systems, that directed against an intracellular, microsomal constituent; the other, which involves thyroglobulin, is not only organ specific, but is much more constantly demonstrable in human "auto-immune thyroiditis."

The matter seemed to us to warrant further attention. A statistically valid comparison between histology and serology in conditions only sometimes associated with auto-immunity was needed to determine to what extent antibody tests can predict pathological changes, and whether they should be accorded clinical significance. It might also throw light on a more fundamental problem: Is auto-immunity an unimportant epiphenomenon of thyroid damage or overstimulation, due to leakage of antigenic components and antibody production by the peripheral reticulo-endothelial system? Or does the antibody in the blood stream originate from the gland itself and therefore signify a self-destructive invasion by the immune defences?

The results of such a study, based on a retrospective analysis of surgical specimens of thyroid tissue recently submitted for routine histological examination to the Department of Pathology, are reported here.

Material and Methods

The histological material which formed the starting point of the investigation consisted of excised portions of thyroid gland from 54 patients recently operated upon for either diffuse thyrotoxic or adenomatous goitre (19 and 35 cases respectively). Seventy-nine cases were originally selected from the total material available by one of us (R.O.K.S.), the bases of selection being adequacy of sections from each specimen in respect of number and quality and the inclusion of approximately equal numbers in which infiltration with lymphocytes and plasma cells and lymphadenoid collections were (*a*) obvious and of at least moderate degree, and (*b*) absent or negligible. Other histological abnormalities such as Askanazy-cell change in the thyroid epithelium and the presence of multinucleated giant cells were not taken specifically into account, though these were also often present in group (*a*). Fifty-seven of the patients concerned were successfully contacted and proved willing to provide a specimen of blood for immunological study. The sections were then reviewed independently by G.A.S., whose assessment of the extent of lymphoid invasion was found to coincide with the original classification in all except two instances; the cases in dispute were on that account discarded from the analysis. R.H., without knowledge of either the histological or serological findings, subsequently examined the pre-operative clinical and laboratory data in order to confirm that the stated clinical diagnosis had been adequately established; a third case was rejected on the grounds of doubt about the true state of thyroid function before thyroidectomy, leaving a final total of 54. There was evidence of increased thyroid function pre-operatively in all 19 patients with Graves' disease and in nine of those with nodular goitre, the remainder being euthyroid. The three clinical groups each comprised approximately equal proportions with and without lymphocytic infiltration of the gland (Table I).

TABLE I
CLASSIFICATION OF 54 CASES ON BASIS OF PRE-OPERATIVE DIAGNOSIS AND HISTOLOGICAL INFILTRATION WITH LYMPHOCYTES

Diagnosis	No. of Cases (Total)	No. with Appreciable Lymphocytic Invasion	No. with Negligible Lymphocytic Invasion
Adenomatous goitre (euthyroid)	26	14	12
Adenomatous goitre (hyperthyroid)	9	5	4
Diffuse thyrotoxic goitre (Graves')	19	10	9
Totals	54	29	25

Antibody to thyroglobulin was estimated by an adaptation of the tanned red cell agglutination technique (Boyden, 1951), the details of which have been described elsewhere (Owen and Smart, 1958); the cells were coated with antigen by treatment with a suitably diluted and buffered high-speed supernate of a homogenate of human thyroid gland. Standard complement-fixation technique was used for estimating the complement-fixing antibody, following in essentials that employed by Roitt and Doniach (1958); antigen was prepared from thyrotoxic tissue and was subjected to low-speed centrifugation only. All serological tests were carried out by one of us (S.G.O.) without knowledge of the histological classification.

Histological and serological data were then compared, with the results summarized below. Though antibody levels were estimated quantitatively, only the presence or absence of each antibody has been considered for purposes of analysis. This was dictated by the retrospective nature of the study, serology having been carried out at varying intervals after partial thyroidectomy. The high titres characteristic of active Hashimoto's disease are known to drop sharply within a year of thyroidectomy (Owen and Smart, 1958), so it is likely that observed levels among the present patients variably underestimate those existing at the time of operation. (By chance, the sera of five patients had been submitted to the laboratory before thyroidectomy, and of these two were positive; though still positive on re-testing during the study, the antithyroglobulin concentration had fallen from 7 to 4 logarithmic units in one and from 10 to 2 units in the other over respective periods of three and 12 months.) It is possible that depression below the threshold necessary for detection in some cases may have led to the overall frequencies reported here being falsely low, but any such effect will be small since in our experience antibody, to thyroglobulin at least, rarely disappears entirely.

Results

Considering the two immune systems together, there was a highly significant positive association between auto-immunity and round-celled infiltration (Table II). When type of antibody is taken into account, the correlation is seen to depend almost entirely on that to thyroglobulin; though positive complement-fixation reactions were somewhat commoner in the lymphadenoid group, the difference is not statistically significant (Table III).

TABLE II
RELATION BETWEEN LYMPHOCYTIC INFILTRATION AND THYROID AUTO-IMMUNITY

Histological Assessment of Lymphocytic Infiltration	No. of Cases	No. Showing Auto-immunity (to Either or Both Antigens)
(*a*) Appreciable	29	20 (69%)
(*b*) Negligible	25	3 (12%)

$\chi^2_c = 15{\cdot}2$ $P < 0{\cdot}001$

TABLE III
RELATION BETWEEN LYMPHOCYTIC INFILTRATION AND INDIVIDUAL ANTIBODIES

Histological Assessment of Lymphocytic Infiltration	No. of Cases	No. with Antibody to Thyroglobulin	No. with Antibody to Complement-fixing (Microsomal) Antigen
(*a*) Appreciable	29	17 (59%)	8 (28%)
(*b*) Negligible	25	1 (4%)	2 (8%)
		$\chi^2_c = 15{\cdot}6$ $P < 0{\cdot}001$	$\chi^2_c = 2{\cdot}2$ $P < 0{\cdot}20 > 0{\cdot}10$

TABLE IV
RELATION BETWEEN DIAGNOSTIC CLASSIFICATION AND AUTO-ANTIBODIES

Classification on Basis of Diagnosis	No. of Cases	No. with Antibody to Thyroglobulin		No. with Antibody or Complement-fixing (Microsomal) Antigen	
(*a*) Adenomatous goitre (euthyroid)	26	8 (31%)	$\chi^2_c \sim 0$	2 (7·7%)	$\chi^2_c = 4{\cdot}5$ $P < 0{\cdot}2 > 0{\cdot}1$
(*b*) Adenomatous goitre (hyperthyroid)	9	3 (33%)		1 (11%)	
(*c*) Diffuse thyrotoxic goitre (Graves')	19	7 (37%)		7 (37%)	
(*a*)+(*b*) All adenomatous goitres	35	11 (31%)	$\chi^2_c \sim 0$	3 (8·6%)	$\chi^2_c = 4{\cdot}8$ $P < 0{\cdot}05$
(*c*) Diffuse thyrotoxic goitre (Graves')	19	7 (37%)		7 (37%)	
(*a*) Adenomatous goitre (euthyroid)	26	8 (31%)	$\chi^2_c \sim 0$	2 (7·7%)	$\chi^2_c = 2{\cdot}6$ $P < 0{\cdot}2 > 0{\cdot}1$
(*b*)+(*c*) All hyperthyroid goitres	28	10 (36%)		8 (29%)	

Table IV relates frequency of auto-immunity to clinical diagnosis. As expected from the equal proportions of the two histological patterns, the incidence of thyroglobulin antibody did not vary between the three clinical groups. The presence of the complement-fixing antibody, on the other hand, was significantly related to the diagnosis of Graves' disease.

Discussion

Our findings are fully consistent with the view that thyroglobulin antibody in the circulation is derived from reticulo-endothelial elements within the gland itself (Burnet, 1959). They cannot indeed be interpreted otherwise, unless it be postulated that lymphocytic invasion is secondary to damage by antibody manufactured elsewhere and borne to the gland in the blood stream, a hypothesis which is unlikely in view of experimental evidence that exogenous antibody to thyroglobulin is harmless to intact thyroid tissue *in vivo* (Roitt and Doniach, 1958) and in tissue culture (Pulvertaft, Doniach, Roitt, and Hudson, 1959). It is true that one of our cases giving a serological reaction to thyroglobulin failed to show obvious lymphadenoid change on histology, but it is possible that this was an artefact introduced by limitations inherent in the retrospective method, in particular our inability to examine representative sections from all areas of the gland.

If antibody in the circulation does in fact represent spill-over from the auto-immune reaction in thyroid tissue, its presence and concentration will vary with the local balance between antibody and antigen. Thus the dense, uniform cellularity and the paucity of colloid characteristic of active Hashimoto's disease bespeak antibody predominance and explain the high titres associated with the condition. While the present overall incidence of thyroglobulin antibody was determined by histological selection and does not therefore reflect the natural frequency in the clinical groups studied, the observed frequency amongst the patients showing lymphoid changes (about 60%) might be expected since the changes were usually only moderate and total fixation of antibody by antigen may often have occurred within the gland. At the moment these considerations remain to some extent speculative ; we hope to obtain more definitive evidence by extending the method to include approximate quantitative assessment of cellularity and tissue volume.

Failure to establish a similar association in the case of the complement-fixation antibody does not deny that one may exist ; the site and manner of formation remain uncertain. But the complement-fixing property *ipso facto* invites speculation as to whether this antibody may not belong to a different order of immune defence, adapted to function in the circulation rather than the tissues. The homologous antigen, which has an intracellular distribution resembling that of the microsomes (Holborow, Brown, Roitt, and Doniach, 1959), is abundant only in thyrotoxic goitres (Trotter, Belyavin, and Waddams, 1957 ; Belyavin and Trotter, 1959), which presumably accounts for the higher incidence of the antibody in Graves' disease. The observation that hyperthyroidism complicating thyroid adenoma does not confer a similar increase in incidence is of some interest, since it suggests that overactivity of pituitary thyrotropin rather than hyperthyroidism *per se* may be responsible for exposure of this auto-antigen.

We are grateful to those physicians and surgeons of the Royal Victoria Infirmary who allowed us to study patients under their care. Our thanks are also due to Mrs. R. T. Johnson for her skilled assistance with the haemagglutination method.

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THE RELATION OF SERUM URIC ACID TO HAEMOGLOBIN LEVEL IN PATIENTS WITH CARDIAC AND RESPIRATORY DISEASE

BY

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An investigation was made of the serum uric acid in 130 male patients with long-standing cardiac or pulmonary disease. The mean serum uric acid was 4.66 mg. per 100 ml. with a standard deviation of ±0.99.

The serum uric acid increased parallel with a rise in the haemoglobin level. The coefficient of correlation was +0.5, which was nearly six times the standard error.

The frequency of hyperuricaemia (over 6 mg. per 100 ml.) was 11 times in 47 patients with a haemoglobin level above 110% as compared with twice in 83 patients when the haemoglobin level was below this figure.

The highest levels of serum uric acid were seen in patients with cyanotic congenital heart disease.

Two of the patients with hyperuricaemia gave a suggestive history of gout, and this was believed to have been caused by the secondary polycythaemia.

Serum uric acid levels were significantly higher, as were the haemoglobin concentrations, in the obese compared with the patients who were not obese.

This study originated from a clinical impression that in certain patients a moderate elevation of the haematocrit or haemoglobin concentration was associated with a high average, or above normal, level of serum uric acid (Goldthwait, Butler, and Stillman, 1958; Beckett and Lewis, 1960). These subjects were not known to have polycythaemia rubra vera, a condition in which hyperuricaemia is not uncommon (Lawrence, Berlin, and Huff, 1953).

The purpose of this investigation was to test the validity of this impression by relating the serum uric acid to different levels of the haemoglobin. The patients studied had chronic cardiac or respiratory disorders which are known at times to have an accompanying secondary polycythaemia, and hence could be expected to show a wider range of haemoglobin readings than normal subjects. At the same time the opportunity was taken to study the frequency of hyperuricaemia in patients with chronic chest disease, and some of the factors which appeared to influence the level of the serum uric acid.

Material and Methods

The serum uric acid was measured in selected male patients with long-standing chest disease attending the Brompton Hospital. The method of estimation was that of Folin (1922) modified by Folin and Trimble (1924). The range of normal accepted was 1.5 to 6.0 mg. per 100 ml., and the standard deviation of duplicated tests by this method was ±5%. Individual values of the serum uric acid were correlated with the haemoglobin level, type of chest disease, and the presence of obesity. Patients were excluded from the study if they were treated with anticoagulant therapy, salicylates, or corticosteroids at the time of the test. They were also not included if they had chronic renal disease with proteinuria, primary polycythaemia, acute pneumonia, or were having radiation therapy.

The haemoglobin level was measured by the oxyhaemoglobin method using a photoelectric colorimeter, with a neutral grey solution as a standard. A haemoglobin value of 100% was taken to equal 14.6 g. per 100 ml.

Results

One hundred and thirty patients were investigated, and their mean serum uric acid was 4.66 mg. per 100 ml. with a standard deviation of ±0.99; the values ranged from 2.8 to 8.5 mg. per 100 ml. The correlation of the serum uric acid with the percentage haemoglobin is shown in Table I and Fig. 1, and it will be seen that there is an increase in average values with rise of haemoglobin concentration. The coefficient of

TABLE I
SERUM URIC ACID RELATED TO HAEMOGLOBIN LEVEL IN 130 PATIENTS WITH CHEST DISEASE

	Percentage Haemoglobin				
	<90	90–	100–	110–	>120
No. of patients ..	10	31	42	34	13
Mean serum uric acid (mg./100 ml.)	3·84	4·1	4·7	5·08	5·4
No. with hyperuricaemia	—	—	2 (5%)	7 (21%)	4 (31%)

correlation was +0.5, and was of significance as it was nearly six times the standard error. The frequency of hyperuricaemia is also given in Table I for the different haemoglobin ranges, and it appears that serum uric acid values above normal were uncommon when the haemoglobin concentration was below 110%.

All the 130 male patients had symptoms or signs of chronic cardiac or respiratory disease, and most required admission to hospital for treatment. Within this group the highest values of haemoglobin and serum uric acid were seen in those with cyanosis, especially if caused by congenital heart disease, or cor pulmonale. The clinical features of the 13 patients with hyperuricaemia are shown in Table II. Two of the patients with hyperuricaemia and a high haemoglobin value gave a history of joint pain which clinically resembled gout.

TABLE II
CLINICAL STATE OF 13 PATIENTS WITH HYPERURICAEMIA

Patient	Age	Disease	Serum Uric Acid (mg./100 ml.)	Haemoglobin Level (%)
B. C.	18	Pulmonary stenosis with right to left shunt	8·5	140
P. D.	22	Atrial septal defect, anomalous venous drainage ..	7·5	120
C. H.*	58	Emphysema, cor pulmonale	6·9	128
J. P.	70	Bronchitis, emphysema, coronary disease, obesity ..	6·8	118
R. H.	42	Asthma, obesity	6·7	106
P. W.*	37	Patent ductus with pulmonary hypertension, heart failure, and tuberculosis	6·6	148
S. B.	66	Bronchitis and emphysema ..	6·5	116
E. W.	59	Emphysema with cyanosis ..	6·5	100
H. C.	66	Emphysema, cor pulmonale	6·1	112
F. B.	57	Cor pulmonale, emphysema	6·0	120
J. L.	53	Bullous emphysema	6·0	118
G. Y.	55	Emphysema with cyanosis ..	6·0	115
W. B.	52	Chronic rheumatic carditis ..	6·0	112

* Gout.

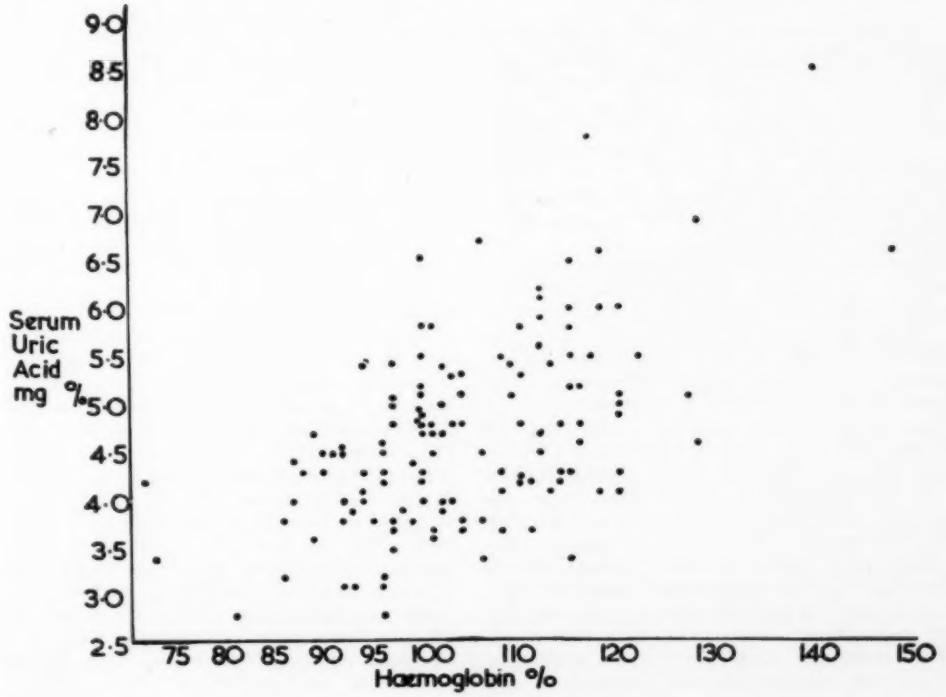

FIG. 1

The relation of the serum uric acid and haemoglobin level to body weight is shown in Table III. The diagnosis of obesity was based on the known weight and appearance of the patient, taking into account the sex, height, and age. In only 89 patients were the clinical details

TABLE III
SERUM URIC ACID AND HAEMOGLOBIN LEVELS IN 85 PATIENTS RELATED TO BODY WEIGHT

	Overweight	Not Overweight
No. of patients	37	52
Average serum uric acid level (mg./100 ml.)	5·0	4·23
Standard deviation	0·88	0·67
Average haemoglobin level ..	106	99·4
Standard deviation	11	9·0

such that an assessment could be made of the presence or absence of obesity. The mean values of the serum uric acid and haemoglobin were significantly higher in the overweight patients than in those not overweight ($P<0.01$). There was no increase of the serum uric acid with age.

Discussion

The study showed that there was an association between the haemoglobin level in its higher ranges and the serum uric acid. High haemoglobin concentrations may reflect a number of conditions such as an absolute increase in red cells due to secondary polycythaemia, a relative polycythaemia caused by a contraction of the plasma volume (Berlin, Lawrence, and Gartland, 1950), or a shift in the distribution of red cells (Blum and Zbar, 1959).

There are several possible reasons why an absolute or relative increase in haemoglobin concentration may be associated with a corresponding rise in the serum uric acid. First, the augmented production of red cells and nucleoproteins in secondary polycythaemia leads to an increased total of uric acid in the body, and hence serum levels will rise to an extent which depends on renal excretion. Second, in patients with compensatory polycythaemia a high haemoglobin level is the consequence of anoxia, which may also interfere with cardio-renal function, and so cause retention of uric acid. Third, in relative polycythaemia the plasma volume is correspondingly decreased, and a proportionate reduction may also be seen in patients with an increased red cell mass (Berlin *et al.*, 1950; Lawrence *et al.*, 1953; Blum and Zbar, 1959). This diminution in available solvent might be expected to contribute to a secondary rise in the concentration of serum uric acid. Finally there is the factor that when respiratory disease is associated with secondary polycythaemia heart failure is often present as well (Cournand, 1950; Wilson, Borden, and Ebert, 1951), and these patients often need diuretic therapy which may itself cause the serum uric acid to rise.

The average serum uric acid of the men studied may not be representative of those with chronic cardiac or respiratory disease, since there was a bias towards patients with a high haemoglobin level. The incidence of hyperuricaemia (10%) compared with the 3 to 6% expected in a random selection (Gertler, Garn, and Levine, 1951; Goldthwait *et al.*, 1958). Persistently high levels of serum uric acid were infrequent in those with long-standing respiratory disease in the absence of a significantly raised haemoglobin, and it is not unexpected that patients with anoxia, cyanosis, and cor pulmonale should have higher average serum uric acid levels than those with healed tuberculosis, minor respiratory disease, or normal subjects.

Hyperuricaemia, on the other hand, is not uncommon in cardiological practice, for it is prevalent in those with premature coronary artery disease (Gertler *et al.*, 1951). The highest values of serum uric acid in the present study were observed with congenital heart disease. The view that an elevated serum uric acid is mainly a reflection of an increased red cell production may apply especially to cyanotic congenital heart disease since secondary polycythaemia is most extreme in this condition (Whitby and Britton, 1950). The resulting hyperuricaemia is clinically important since secondary gout may occur as shown by Yü, Wasserman, Benedict, Bien, Gutman, and Stetten, 1953), and probably by two of the patients in this study.

Significantly higher haemoglobin levels were seen in the obese patients. It is known that obesity can cause cardiorespiratory disease with anoxia and so lead to a compensatory increase in blood volume (Arnott, 1960), but this only occurs in extreme examples. Polycythaemia may also be present in patients with obesity even though the oxygen tension is normal (Weil, 1955). Certain workers, such as Huff and Feller (1956), have shown that the mass of circulating red cells is significantly related to fat as well as lean tissue. It is thus tempting to ascribe, at least in part, the higher average serum uric acid of the obese patient with cardiorespiratory disease to an increased production of red cells as compared with those not overweight. This might also explain why higher average serum uric acid levels

are seen in the normal endomorphic subject, and those of similar build with coronary artery disease (Gertler *et al.*, 1951), and diabetes mellitus (Beckett and Lewis, 1960) as compared with ectomorphs.

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THE DETERMINATION OF MAGNESIUM IN BIOLOGICAL FLUIDS AND TISSUES BY FLAME SPECTROPHOTOMETRY

BY

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A flame spectrophotometric method for estimating magnesium in biological fluids and tissues is described. It may be applied to the Zeiss spectrophotometer PMQ II with flame attachment. Interferences are small, and recoveries from plasma and urine and from ashed faeces, soft tissue, and bone are satisfactory. Calcium can be measured on the same dilutions of plasma and urine and faecal ash. Sodium, potassium, calcium, and magnesium can be estimated on the same dilutions of soft tissue and bone ash.

The plasma magnesium concentration of normal subjects falls within a narrow range. Differences between mean normal values previously reported may be partly attributed to the magnesium salt used in preparation of standard solutions.

Due to the lack of suitable analytical methods very little is known of the role of magnesium in disease in man. Available methods are either tedious or inaccurate, and plasma magnesium concentrations are estimated in clinical laboratories as rarely as was potassium before the advent of the flame photometer.

Flame photometric estimation is made difficult by the much higher excitation potential of magnesium compared with that of sodium, potassium, and calcium (Mavrodineanu and Boiteux, 1954). The magnesium oxide band at 371 $m\mu$ is more easily excited than the atomic line at 285.2 $m\mu$, but has been used only at the expense of a cumbersome internal standard procedure to avoid interference by sodium (Teloh, 1958) or of separate estimation and adjustment of the sample sodium concentration (Wacker and Vallee, 1957). Unfortunately the flame background at 285.2 $m\mu$ is extremely complex and a further complication is the presence of a sodium line at 285.3 $m\mu$ (Mavrodineanu and Boiteux, 1954). Hence a very narrow band width must be selected by the monochromator in order to isolate the magnesium emission. This may lead to inadequate sensitivity. Davis (1955), Manna, Strunk, and Adams (1957) and van Fossan, Baird, and Tekell (1959) used an organic solvent to enhance emission, and Kapuscinski, Moss, Zak, and Boyle (1952) attained adequate sensitivity by concentrating the sample after wet oxidation.

This paper describes a simple flame spectrophotometric method which overcomes these difficulties.

EXPERIMENTAL

The flame spectrophotometer previously described (MacIntyre and Davidsson, 1958) was used with an oxyacetylene flame. Oxyhydrogen gave insufficient sensitivity. The oxygen pressure was set at 25 lb./sq. inch, the slit width at 0.02 mm., and the wavelength selector at about 285 $m\mu$. The peak of the magnesium line was located by noting the maximum deflection on the galvanometer when a concentrated solution (50 mEq./l.) of a magnesium salt was sprayed, while rotating the wavelength selector through 284 to 286 $m\mu$. The acetylene pressure was then adjusted to produce the greatest possible value of the ratio:

$$\frac{\text{emission due to magnesium}}{\text{emission due to flame background}}$$

when a solution of 0.1 mM-magnesium salt was sprayed. Sensitivity was judged adequate when this ratio was 1.0 or greater with a 0.1 mM-magnesium solution. The instrument was then ready for taking readings.

Galvanometer deflections of each unknown solution were bracketed between those of the appropriate adjacent standards and readings taken in duplicate. The magnesium content was calculated by interpolation.

Determination of Interference Effects

All solutions were made with deionized distilled water and were prepared from AnalaR chemicals (British Drug Houses, Poole, England) with the exception of the magnesium and calcium stock solutions. The calcium

solutions were made from " specpure " calcium carbonate (Johnson, Matthey & Co. Ltd., London) dissolved in the minimum volume of concentrated HCl. The magnesium stock solution was prepared from " specpure " magnesium sulphate. Polythene containers were used to store all solutions.

In studying interference effects solutions of the same magnesium concentration were prepared containing increasing amounts of the constituent whose effect was being examined. Galvanometer deflections produced by these solutions were recorded. A blank solution in which magnesium was replaced by water, but identical in all other respects, was sprayed in every case.

Method for Determination of Magnesium in Biological Fluids and Tissues

Stock Solutions.—The following were used :

Magnesium Stock Solution.—A solution of 25 mM-$MgSO_4.7H_2O$, of which the concentration was checked by titration with disodium ethylenediaminetetra-acetate which had been standardized with calcium stock solution and with standard zinc acetate solution.

Mixed Salt Solution A.—30 mM-KCl, 5 mM-K_2SO_4, 1.4 M-NaCl, 50 mM-KH_2PO_4.

Mixed Salt Solution B.—50 mM-KCl, 5 mM-K_2SO_4, 50 mM-NaCl, 150 mM-KH_2PO_4.

Mixed Salt Solution C.—2 mM-KCl, 12 mM-NaCl, 310 mM-$NH_4H_2PO_4$.

Calcium Stock Solution I.—25 mM-$CaCl_2$.

Calcium Stock Solution II.—250 mM-$CaCl_2$.

Perchloric Acid.—$HClO_4$ 60% (w/w).

Hydrochloric Acid.—HCl (S.G. 1.18).

Phosphoric Acid.—H_3PO_4 (S.G. 1.75).

Phosphate Solution—44.4 mM-KH_2PO_4.

Sodium Solution.—200 mM-NaCl.

Standards for Plasma, Urine, and Faeces.—Solutions were prepared by adding 10 ml. of mixed salt solution A, 10 ml. calcium solution I, and an appropriate volume of magnesium solution to 700 ml. of water in a litre flask. Perchloric acid (50 ml.) was now added and the volume made up to 1 litre. A range of standards at intervals of 0.05 mM was prepared from 0.05 to 0.40 mM with respect to magnesium concentration.

Standards for Soft Tissues.—Standard solutions were prepared to cover the range 0.125–1.00 mM with respect to magnesium concentration at intervals of 0.125 mM. 10 ml. of salt solution B, 2 ml. of calcium solution I, and an appropriate volume of magnesium solution were added to approximately 700 ml. of water in a litre flask. HCl (100 ml.) was added and the volume made up to 1 litre.

Standards for Bone.—Standards to cover the range 0.25–3.0 mM with respect to magnesium concentration were prepared. An appropriate volume of stock standard magnesium solution, together with 100 ml. calcium solution II, 100 ml. mixed salt solution C, and 100 ml. HCl, was made up to 1 litre.

Combined Diluting and Deproteinizing Fluids.—(1) Phosphate solution (10 ml.) was diluted to approximately 700 ml. in a volumetric flask, mixed with 55.5 ml. $HClO_4$, and made up to 1 litre. This solution was used for plasma and urine analysis.

(2) Phosphate solution (10 ml.) and sodium solution (25 ml.) were diluted to approximately 700 ml. in a volumetric flask. Perchloric acid (55.5 ml.) was added and the volume made up to 1 litre. This solution was used for analysis of faecal ash.

(3) H_3PO_4 (1 ml.) was added to HCl (100 ml.) and the volume made up to 1 litre. This solution was used for soft tissue and bone analysis.

Procedure

Plasma or Serum.—One volume of plasma or serum is added drop by drop to 9 volumes of combined diluting and deproteinizing fluid 1. The tube is capped with " parafilm " (A. Gallenkamp & Co. Ltd., London, E.C.2) and the contents mixed by gently inverting the tube several times. It is then centrifuged and the clear supernatant used.

Urine.—Urine may be diluted similarly to plasma, or dilution increased, e.g., 1 in 20 as necessary. Twenty-four-hour urine specimens should be collected in a Winchester bottle containing 10 ml. concentrated HCl in order to prevent precipitation of magnesium.

Faeces.—Specimens were homogenized and a sample dried at 105° for 16 hours. A known weight (approximately 1.0 g.) was ashed in a platinum crucible at 400°. The ash was dissolved in N-HCl (200 ml. HCl per gram of dry faeces). The solution was thoroughly shaken to ensure complete dissolution. The solution was further diluted as required with combined diluting and deproteinizing fluid 2. Further ten- or twenty-fold dilution was normally appropriate.

Soft Tissues.—Soft tissues were treated as previously described (MacIntyre and Davidsson, 1958). The ash was dissolved in diluting fluid (4 ml. per 20 mg. tissue powder).

Bone.—Bone was dried at 105° for 16 hours. It was defatted by immersing for eight hours in changes (3 × 5 ml.) of a mixture of equal volumes of diethyl ether and light petroleum (B.P. 40°–60°). The sample was again dried at 105° for six hours, weighed, and ashed in a platinum crucible at 420°. The ash was dissolved in three or four drops of HCl, taken to dryness and redissolved in diluting fluid 3 (40 ml. per 200 mg. dried fat-free bone).

RESULTS

Interference Effects

Flame Background.—Interference from flame background was studied at 285.2 mμ and also at the oxide bands at 371 and 383 mμ. Results are summarized in Table I. The background interference is least at 285.2 mμ.

Sodium Emission at 285.3 mμ.—It is essential that the maximum resolution be employed to minimize

TABLE I
EMISSION DUE TO SODIUM AND MAGNESIUM

	285·2 mμ	371 mμ	383 mμ
Ratio $\frac{\text{Mg emission (0·1 mM-Mg)}}{\text{flame background}}$	1·0–1·5	0·03	0·03
*Na interference (0·1 mM-Mg; 20 mM-Na)	2	500	200

*Na interference was expressed by the ratio:

$$\frac{\text{emission due to sodium}}{\text{emission due to magnesium}} \times 100$$

the interference due to the sodium emission at 285.3 mμ. The emission spectrum of a solution containing 0.1 mM-magnesium and 20 mM-NaCl solutions was studied. These concentrations are similar to the proportions of magnesium and sodium in plasma. It was found that the emission due to sodium was 2% of that due to magnesium. The emission of magnesium at the oxide bands at 371 and 383 mμ was also studied. Comparison of the emissions due to magnesium and to sodium are summarized in Table I.

Other Inorganic Constituents.—Potassium chloride, calcium chloride, ammonium sulphate, ammonium dihydrogen phosphate, and potassium dihydrogen phosphate in concentrations up to 20 mM had no effect on Mg emission at 285.2 mμ.

HCl, $HClO_4$, and H_3PO_4 in the concentrations used were without effect on magnesium emission.

Organic Constituents.—Samples of pooled human plasma were ashed at 400° for 16 hours. Magnesium was estimated on the ash and the results compared with those obtained by direct estimation (Table IV). Agreement was good. Hence organic constituents present in plasma are without effect on magnesium emission.

Recovery Experiments

Recoveries of magnesium added to plasma and urine are shown in Tables II and III respectively. Pooled human plasma of low magnesium content was prepared by passing through a column of "zeocarb 225" cation exchange resin (Permutit Co. Ltd., London, W.4). The calcium, potassium, and sodium contents of the plasma were adjusted to 5.0, 5.0, and 140.0 mEq./l. respectively, by adding appropriate amounts of $CaCl_2$, KCl, and NaCl. Varying amounts of magnesium (as magnesium sulphate) were added to samples of the pooled plasma. Magnesium was then estimated in each sample, determinations being carried out in quadruplicate.

TABLE II
RECOVERY OF MAGNESIUM ADDED TO PLASMA

Sample No.	Obtained (mEq./l.) (*a*)	Expected (mEq./l.) (*b*)	Difference (mEq./l.) (*a*−*b*)	100*a*/*b*
1	0·32	—	—	—
2	0·79	0·82	−0·03	96·3
3	1·33	1·32	+0·01	100·8
4	1·82	1·82	0	100·0
5	2·37	2·32	+0·05	102·2
6	2·89	2·82	+0·07	102·5
7	3·48	3·32	+0·16	104·8
8	3·91	3·82	+0·09	102·4
Mean (±S.E.)			+0·050 (±0·024)	101·3 (±1·01)

TABLE IIIa
RECOVERY OF MAGNESIUM ADDED TO RAT URINE

Sample No.	Obtained (mEq./l.) (*a*)	Expected (mEq./l.) (*b*)	Difference (mEq./l.) (*a*−*b*)	100*a*/*b*
1	0·02	—	—	—
2	1·06	1·02	+0·04	103·9
3	2·08	2·02	+0·06	103·0
4	3·16	3·02	+0·14	104·6
5	4·18	4·00	+0·18	104·5
6	4·81	5·00	−0·19	96·2
7	6·04	6·00	+0·04	100·7
8	7·13	7·00	+0·13	101·9
9	8·11	8·00	+0·11	101·4
Mean (±S.E.)			0·064 (±0·040)	102·0 (±0·98)

TABLE IIIb
RECOVERY OF MAGNESIUM ADDED TO RANDOM SPECIMENS OF HUMAN URINE

Sample No.	Obtained (mEq./l.) (*a*)	Expected (mEq./l.) (*b*)	Difference (mEq./l.) (*a*−*b*)	100*a*/*b*	Description*
1	10·94	11·15	−0·21	98·1	Calcium (30 mEq./l.)
2	2·10	1·99	+0·11	105·5	Glucose (300 mg./100 ml.)
3	3·43	3·47	−0·04	98·8	Protein (0·5 g./100 ml.)
4	7·12	7·26	−0·14	98·1	Bile + urobilinogen
5	3·56	3·44	+0·12	103·5	Normal
6	5·52	5·27	+0·25	104·7	,,
7	9·82	9·96	+0·14	98·6	,,
8	4·49	4·48	+0·01	100·2	,,
9	3·84	3·85	−0·01	99·7	,,
10	4·24	4·37	−0·13	97·0	,,
Mean (±S.E.)			−0·018 (±0·047)	100·4 (±0·96)	

*Abnormal components of urine are shown under the heading "Description."

Urine of low magnesium content obtained from magnesium-deficient rats was used for recovery experiments (Table IIIa). In addition a constant amount of magnesium was added to human urine specimens of differing composition (Table IIIb).

Varying amounts of magnesium were added to dried homogenized faeces, dried muscle which had previously been defatted and homogenized, and to dried defatted bone. Recovery of magnesium added to samples of each is shown in Tables V, VI, and VII respectively.

TABLE IV

COMPARISON OF PLASMA MAGNESIUM ESTIMATIONS BEFORE AND AFTER ASHING AT 400°

Sample No.	After Ashing (mEq./l.) (*a*)	Before Ashing (mEq./l.) (*b*)	Difference (mEq./l.) (*a*−*b*)	100*a*/*b*
1	1·56	1·56	0	100
2	1·56	1·56	0	100
3	1·56	1·62	−0·06	96·3
4	1·75	1·81	−0·06	96·7
5	1·47	1·47	0	100
6	1·50	1·50	0	100
7	1·60	1·60	0	100
8	1·74	1·71	+0·03	101·8
9	1·25	1·38	−0·13	90·6
10	1·31	1·38	−0·07	94·9
Mean (±S.E.)			−0·029 (±0·015)	98·03 (±1·07)

TABLE V

RECOVERY OF MAGNESIUM FROM DRIED FAECES

Sample No.	Obtained (μEq./g.) (*a*)	Expected (μEq./g.) (*b*)	Difference (μEq./g.) (*a*−*b*)	100*a*/*b*
1	368	370	−2	99·5
2	430	400	+30	107·5
3	132	140	−8	94·3
4	504	496	+8	101·6
5	464	430	+34	107·9
6	166	170	−4	97·6
7	230	240	−10	95·8
8	302	294	+8	102·7
Mean (±S.E.)			7 (±5·9)	100·9 (±1·8)

Normal Plasma Values

Blood from 76 healthy adults (either laboratory personnel or blood donors) was collected into bottles containing sodium heparin. The magnesium concentration was estimated in duplicate. The mean ± the S.E. was 1.66 ± 0.01 mEq./l. The distribution is shown in the histogram (Fig. 1). No sex differences in magnesium concentration were found. The narrow range in which the magnesium values in plasma of healthy adults falls is striking.

After this work had been completed a Zeiss spectrophotometer PMQ II with single monochromator M4QII or double monochromator MM12 (Carl Zeiss, Oberkochen, Germany) with flame attachment became available. The burner was modified by enlarging the fuel inlet to 1.3 mm. An oxyacetylene flame was used at pressures of 250 mm. water and 0.37 kg./sq. cm. for acetylene and oxygen respectively. Magnesium estimations were made at 285.2 mμ with a slit width of 0.0175 mm. (single monochromator) or 0.02 mm. (double monochromator)

TABLE VI

RECOVERY OF MAGNESIUM FROM DRIED FAT-FREE MUSCLE

Sample No.	Obtained (mEq./kg.) (*a*)	Expected (mEq./kg.) (*b*)	Difference (mEq./kg.) (*a*−*b*)	100*a*/*b*
1	121·3	123·6	−2·3	98·1
2	140·9	141·6	−0·7	99·5
3	124·2	127·2	−3·0	97·6
4	135·4	140·2	−4·8	36·6
5	125·0	127·4	−2·4	98·1
6	137·6	135·0	+2·6	101·9
7	136·6	136·6	0	100·0
8	148·0	145·6	+2·4	101·6
Mean (±S.E.)			−1·025 (±0·92)	99·2 (±0·7)

TABLE VII

RECOVERY OF MAGNESIUM FROM DRIED DEFATTED BONE

Sample No.	Obtained (mEq./kg.) (*a*)	Expected (mEq./kg.) (*b*)	Difference (mEq./kg.) (*a*−*b*)	100*a*/*b*
1	408	425	−17	96·0
2	445	464	−19	95·9
3	450	428	+22	105·0
4	479	472	+7	101·5
5	435	418	+17	104·1
6	478	465	+13	102·8
7	468	480	−12	97·5
8	555	528	+27	105·1
9	478	468	+10	102·1
10	531	530	+1	100·2
11	532	525	+7	101·3
12	602	595	+7	101·2
Mean (±S.E.)			+5·25 (±4·25)	101·1 (±0·92)

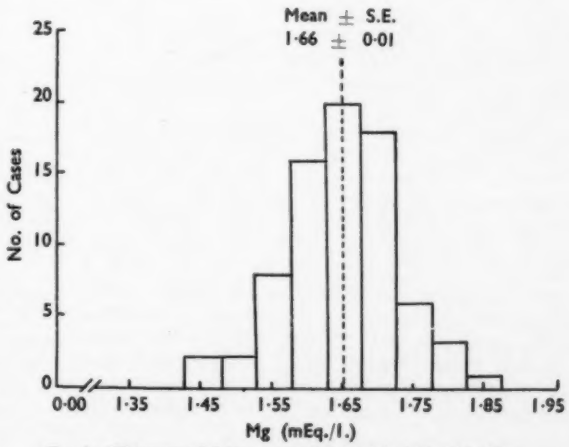

FIG. 1.—Histogram of plasma magnesium levels in 76 healthy adults.

and the instrument adjusted to full sensitivity. Interference effects were almost identical with those described and the instrument is entirely satisfactory for the estimation of magnesium in plasma, urine, faeces, soft tissues, and bone. Background interference is least with the double monochromator MM12.

DISCUSSION

The difficulty in estimating the concentration of magnesium from its emission at 285.2 mμ arises from several sources. The excitation potential of the magnesium line at 285.2 mμ is 4.3 eV compared with values of 2.1, 1.6, and 2.9 for the emission of sodium at 589 mμ, potassium at 766.5 mμ, and calcium at 422.7 mμ respectively. It is therefore necessary to use a hot flame such as oxyacetylene to obtain sufficient sensitivity. Further, even the emission obtained with an oxyacetylene flame is small and requires a photomultiplier for its detection and measurement.

A monochromator of high resolution is necessary to separate the emission of magnesium at 285.2 mμ from the adjacent sodium emission at 285.3 mμ, and from the complex flame background in this region of the spectrum.

The dilutions of muscle and bone ash described are suitable for the simultaneous determination of sodium, potassium, calcium, and magnesium.

Phosphate, sulphate, calcium, and potassium in concentrations encountered in biological fluids were without detectable influence on magnesium emission. Interference due to sodium was 2% or less. Sodium, potassium, calcium, phosphate, and sulphate were incorporated in the standards to allow for any complex interference effects from the presence of mixed salt undetected from interference studies of individual compounds. Excess phosphate was added to allow simultaneous measurement of calcium (MacIntyre, 1957).

Table VIII summarizes some recent values quoted for normal plasma magnesium. A difference of about 25% in the mean value obtained is evident, and may be attributed in part to the source of magnesium used in preparation of standard solutions. Magnesium oxide is not a suitable standard substance: it is difficult to prepare pure and it absorbs carbon dioxide from the air (Mellor, 1923). Complete decomposition of magnesium carbonate only occurs at 900° (Mellor, 1923). The mean normal value obtained using standards prepared from magnesium oxide treated in this way (Davis, 1955; Carr and Frank, 1956) is in agreement with that obtained when the source is pure magnesium metal (van Fc san *et al.*, 1959; Hunter, 1958) or magnesium sulphate as used in this method. Indirect measurement of plasma magnesium by phosphate precipitation gives similar results (Simonsen, Westover, and Wertman, 1947).

Magnesium oxide heated to 110° or 120° would be expected to produce high results and this is seen to be the case (Schachter, 1959; Wacker and Vallee, 1957).

TABLE VIII
MEAN NORMAL VALUES REPORTED FOR PLASMA MAGNESIUM

Author	Method	Standard Magnesium Salt	Mean Normal (mEq. l.)
Davis (1955) ..	Flame spectrophotometric	MgO heated to 800–900° for several hours	1·66
Carr and Frank (1956)	Titration with E.D.T.A.	MgO heated to constant weight	1·72
van Fossan *et al.* (1959)	Flame spectrophotometric	Pure Mg metal	1·67
Hunter (1958)	Matching the colour with eriochrome black T with standard	Mg ribbon	1·69
Simonsen *et al.* (1947)	Phosphate precipitation	—	1·68
Schachter (1959)	Fluorimetric	Reagent grade MgO heated to 110°	2·05
Wacker and Vallee (1957)	Flame spectrophotometric	Reagent grade MgO heated to 120°	2·05
Present series	,, ,,	$MgSO_4$	1·66

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SERUM PROTEINS AND BLOOD ALPHA AMINO NITROGEN IN CEREBRAL PALSY

BY

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The mean serum proteins of 30 children over the age of 3 suffering from cerebral palsy was 7.20 with a standard deviation of 0.49 g. per 100 ml. No difference was found between children with the spastic and athetoid types of cerebral palsy. The electrophoretic pattern of the serum proteins did not differ in the two types, but the alpha 2 globulin diminished significantly with increasing age. There was no difference that could be considered "clinically significant" between the total serum proteins or their electrophoretic pattern in children with cerebral palsy and in four published series of normal children.

The plasma and whole blood alpha amino nitrogen showed no significant difference between the two types of cerebral palsy.

Because there does not appear to be any information about serum proteins in children with cerebral palsy in the literature, the total serum proteins and their electrophoretic pattern in children over 3 years of age with athetoid and spastic types of cerebral palsy were determined. The results in the athetoid and spastic children were compared with each other and with published data in normal children.

Methods

Thirty children (15 boys and 15 girls) were studied between the ages of 3 and 19 years. All the children had been in-patients in this hospital school for at least one month and had been free from infection during this time. The children had all been immunized against diphtheria, pertussis, tetanus, and whooping cough, and had been vaccinated. All blood specimens were taken by venepuncture throughout a four-week period in November and December, 1958. The serum was separated immediately after the clot had retracted and was then stored frozen until the protein estimations were performed. Blood samples for the alpha amino nitrogen determinations were collected in tubes containing dried heparin, but three were haemolysed and discarded. No attempt was made to ensure that blood was drawn in the fasting state since no difference has been found between serum proteins before and after meals (Trevorrow, Kaser, Patterson, and Hill, 1942; Oberman, Gregory, Burke, Ross, and Rice, 1956).

The concentration of protein nitrogen was calculated by the difference between total nitrogen and non-protein nitrogen. The observed nitrogen concentrations were determined by micro-Kjeldahl digestion (Van Slyke and Hugel, 1933) and microdiffusion (Conway and O'Malley, 1942). The total serum proteins were calculated by multiplying the protein nitrogen by 6.25.

The method and apparatus used for filter paper electrophoresis were exactly those described in the instruction manual RIM-5 for the "spinco" model R paper electrophoresis system published by Beckman, California, with the exception that the voltage was kept constant at 80 volts and the division of the components was made by dropping a vertical from the intersection of the Gaussian distribution curves.

The alpha amino nitrogen levels in plasma and whole blood were estimated by the ninhydrin method of Yemm and Cocking (1955) after precipitation of the plasma proteins and haemoglobin with trichloroacetic acid.

Results

The results in the two groups of children with cerebral palsy are tabulated in Table I. There is no significant difference at the 5% level between the means or variances of any of these blood constituents. Similarly there are no significant differences in the variances or means between children of different sexes.

Pearsonian correlation coefficients were estimated between age and each of the blood constituents in Table I. The only significant findings were a negative correlation between age and alpha 2 globulin percentage ($r = -0.3818$; $0.05 > P > 0.02$). The correlation between age and the absolute concentration of alpha 2 globulin was not significant ($r = -0.2179$; $0.20 > P > 0.10$).

In order to make the results comparable, the normal series in Table II were recalculated to eliminate children under 3 years old and to correct

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TABLE I

COMPARISON OF SERUM PROTEINS AND BLOOD ALPHA AMINO NITROGEN LEVELS IN CHILDREN WITH ATHETOID AND SPASTIC TYPES OF CEREBRAL PALSY

	Athetoids			Spastics		
	Mean*	Variance*	No. of Observations	Mean	Variance	No. of Observations
Total protein (g. per 100 ml.)	7·17	0·1405	17	7·25	0·3863	13
Albumin (%)	64·8	13·2688	17	64·5	21·0683	13
α 1 globulin (%)	4·28	0·4075	17	4·28	0·6283	13
α 2 globulin (%)	8·86	2·2625	17	10·26	4·6775	13
β globulin (%)	9·09	2·4650	17	9·02	2·4500	13
γ globulin (%)	13·09	5·8543	17	12·04	4·0308	13
Blood α amino N (mg. per 100 ml.)	5·43	0·4148	16	5·99	0·5540	12
Plasma α amino N (mg. per 100 ml.)	4·62	0·5761	16	5·08	0·7154	12

*There is no significant difference between the means or variances of any of these blood constituents in the two groups of children.

plasma levels to serum levels by eliminating the difference due to fibrinogen. Because no difference was found between spastics and athetoids, all our observations are considered in one group in Table II.

Discussion

The present observations on the serum proteins, their electrophoretic patterns, and the blood alpha amino nitrogen have been performed under particularly standardized conditions. The children were all resident in the same hospital school and were offered the same diet. They had all received the same immunization programme and had been known to be free of infection for at least one month. The specimens were all taken in the same month, thus eliminating the seasonal differences in the serum albumin found by Trevorrow and her colleagues (1942). All the estimations were made in the same laboratories with the same apparatus and by the same technicians within a period of three months. The children differ by age, sex, and diagnosis. Sex and diagnosis have already been shown to make no significant difference to any of these constituents of blood.

Only children over the age of 3 years were studied, partly because it is often difficult to be certain of the type of cerebral palsy earlier. The total serum proteins, which vary in infancy, reach adult levels at about 3 years (Trevorrow and others, 1942), and on electrophoretic studies there are only minor changes in the various fractions after this age (Knapp and Routh. 1949). The only significant change with an increasing age discovered in this series was a fall in the proportion of alpha 2 globulin but not its absolute amount. Knapp and Routh (1949) also noted the fall in the proportion of alpha 2 globulin with rising age but they analysed their results by considering arbitrary age groups. Their results in children 3 to 17 years old were corrected for the presence of fibrinogen and the correlation coefficient ($r = -0.2924$) with age was found to be significant at the 10% level but not at the 5% level. Lubschez'

TABLE II

COMPARISON OF TOTAL SERUM PROTEINS AND THEIR ELECTROPHORETIC PATTERN OF 30 CHILDREN WITH CEREBRAL PALSY WITH PUBLISHED SERIES OF NORMAL CHILDREN*

Source	Total Protein Method	Electrophoretic Method	No. of Subjects	Age of Subjects (Years)	Total Protein (g./100 ml.)	Albumin (%)	Globulin (%)			
							α_1	α_2	β	γ
Present series ..	Micro-Kjeldahl digestion micro-diffusion	Filter paper and "analytrol" densitometer	30	3–19	7·20±0·49 (6·38–8·44)	64·7±4·2 (55·8–73·7)	4·3±0·7 (3·2–5·7)	9·5±1·9 (6·9–13·4)	9·1±1·5 (6·3–12·9)	12·6±2·3 (9·0–17·5)
§Lubschez (1948)		Tiselius and planimetry on Schlieren diagram	27	3–11		†61·4±6·8 (53·2–75·1)	‡5·2±1·7 (2·7–8·8)	10·0±2·2 (6·7–14·9)	‡12·9±2·8 (3·5–17·4)	†10·4±3·9 (4·0–18·1)
§Knapp and Routh (1949)	Micro-Kjeldahl digestion and Nesslerization	As above	38	3–17	7·05±0·52 (6·1–8·3)	‡51·9±3·4 (54·9–67·6)	‡6·0±2·0 (3·2–10·8)	†10·6±2·0 (6·5–18·8)	‡11·9±3·3 (5·8–17·7)	‡9·6±2·7 (4·8–15·6)
Oberman and others (1956)	Biuret	Filter paper and "analytrol" densitometer	42	3–13	7·25±0·40 (6·43–8·19)	‡53·2±4·9 (41·5–65·3)	‡4·9±1·1 (2·8–6·9)	‡11·7±2·3 (6·9–17·4)	‡13·9±2·4 (8·6–22·4)	‡16·3±3·0 (10·1–22·0)
Josephson and Gyllenswärd (1957)	,,	Filter paper and eluting cut strip	24	3–6	7·18±0·57	†62·1±4·4	‡5·1±1·2	9·8±2·1	9·7±1·3	13·3±2·1

* All figures are expressed as the mean plus or minus the standard deviation with the observed range in brackets below. All figures are recalculated to include only data concerning children above the age of 3 years.
† Differs significantly from the present series at the 5% level and ‡ at the 1% level.
§ Original observations made on plasma, but the figures given are corrected here for the absence of fibrinogen.

(1948) results in children aged 3 to 11 were treated in a similar manner, but there was no significant correlation ($r = -0.064$). A falling alpha 2 globulin with increasing age has also been noted by Oberman and others (1956) and by Josephson and Gyllenswärd (1957). After infancy a rising gamma globulin with age has been reported (Knapp and Routh, 1949; Josephson and Gyllenswärd, 1957) but was not observed in the present series nor by Oberman and others (1956), who found that the adult level was reached between 3 and 5 years.

Comparison of the total serum proteins with other recently published series for normal children (Table II) shows that there is no significant difference between the four results despite the fact that two distinctive methods were used. If the four series are combined, the mean of the 134 cases is 7.17 g. per 100 ml. with a standard deviation of 0.48 and an observed range of 6.1 to 8.44 g. per 100 ml. serum.

The total serum proteins in this study have been calculated by multiplying the protein nitrogen by the traditional factor of 6.25 in order that the results might be compared with previously published data. Recently, however, Sunderman, Sunderman, Falvo, and Kallick (1958) have suggested that a factor of 6.54 is more appropriate. Recalculation of the results on the 30 cases of cerebral palsy using the larger factor gives a range of 6.68 to 8.83 and a mean of 7.53 g. per 100 ml. serum.

Filter paper electrophoresis is a simple technique for demonstrating differences in the pattern of the serum proteins, but it is difficult to estimate the various proteins quantitatively by either scanning or elution techniques. The mean deviations of individual methods are surprisingly low (Grassmann, 1956), so that comparisons of different groups using the same laboratory technique with the same apparatus are justifiable; however, comparison between series is unreliable except in the broadest terms. In Table II, the significant differences at the 5 and 1% level between the present series and the published series for normal children are marked, but only the proportion of alpha 1 globulin is consistently different at the 1% level. This fraction is raised in acute infections, particularly within the previous month (Lubschez, 1948). The children used in our series were all under constant medical supervision for at least a month before specimens were collected and were known to be free from any infection during this time. It would be desirable to compare these children with normal children using the same laboratory technique, but it proved impossible to find a group which could be so strictly controlled. The normals tabulated in Table II are thus used for the control group. In view of the above discussion, the difference between the distribution of serum proteins in children with cerebral palsy and normal children is not clinically significant.

The determinations of the protein nitrogen and the alpha amino nitrogen were made by courtesy of Dr. Samuel J. Fomon, of the Department of Pediatrics. The electrophoretic analysis was obtained by the co-operation of Dr. Robert L. Hodges, of the Department of Internal Medicine.

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A COMPARISON OF CALCULATED AND ESTIMATED OSMOLARITIES OF URINE

BY

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Osmolarity has been calculated from estimated cations and organic solutes in 200 samples of urine and compared with that found by direct osmometry.

In three-fifths of the samples the calculated value differs from the found by less than 5%, but in some differences of over 30 m.osmoles/l. and percentage differences of over 20 are found.

Calculated values are frequently too high, this overestimation being particularly marked in alkaline urines, and a change in reaction of the urine in one subject can alter the relationship of the calculated to the true osmolarity in the course of a few hours.

The findings in a small series of samples where the principal anions are estimated suggest that the osmolar activities of the solutes are less in the more alkaline urines than in the more acid ones.

When direct osmometry is not available calculations from the estimated solutes are frequently employed, but are subject to two sources of error acting in opposite directions, underestimation of solutes, giving low values, and overcalculation of osmolar activity, giving high values.

In this paper the found and calculated osmolarities of 200 samples of urine are compared.

Methods

The urine samples were from 20 experiments with 14 subjects. Each experiment gave at least eight samples with wide variation in minute volume and osmolar concentration, these being obtained by variations in fluid intake, or by giving pitressin or chlorothiazide. The scatter of osmolarity in each experiment prevents results at any osmolarity from being weighted by a preponderance of samples from single experiments.

The osmolarity was found from freezing point determinations made with a Fiske osmometer. The following methods of analysis were used: For Na and K, flame photometry; for NH_4 and urea, Conway (1933); for phosphate, Fiske and Subbarow (1925); for creatinine, Jaffe reaction; for Cl, Sanderson (1952), protein, turbidometric; for *p*H, glass electrode.

In the major part of the work the osmolarities were calculated from Na, K, NH_4, urea, creatinine, and protein if present. It was assumed that the three cations are present as chlorides and that their osmotic activity was similar to that of 0.2 M solutions of these salts. Then the osmotic activity is the concentration in m.moles $\times$ 0.925 $\times$ 2 for NaCl and 0.93 $\times$ 2 for KCl and NH_4Cl. Urea and creatinine are assumed to behave as ideal molecules and their osmotic activity to be equal to their molar concentrations. The ionizable and so osmotically active groups are calculated from Van Slyke's formula of g./l. $\times$ 0.243. The Van Slyke factor is for solutions at *p*H 7.4, but variations at other *p*Hs will not significantly affect the total osmolarity since that due to protein is never greater than 10 m.osm./l. The urines in which anions and cations are calculated separately do not contain protein. In a few experiments phosphate and Cl were estimated and the contribution of each ion to the osmotic pressure of the solution was calculated separately, the osmotic coefficient suggested by Yardley (1958) being used. These are as follows:

Na^+	0.95	Cl'	0.92
K^+	0.92	HPO_4''	0.38
NH_4^+	0.92	H_2PO_4'	0.95

Results

The mean differences between calculated and found osmolarities, with the variations given by the standard deviations, are shown in Fig. 1, the samples being grouped according to the found osmolarity.

When calculated values are greater than found (differences positive) the osmolar activity has been overestimated, but, since it is known that the analysis of solutes is incomplete, the overestimation of activity is in fact greater than that shown. When calculated values are less than found (differences negative) there is obvious underestimation of active solutes.

The mean values show increasing overestimation of activity with increasing osmolarity till this reaches 600 m.osmoles/l. At higher osmolarities this decreases and underestimation is apparent.

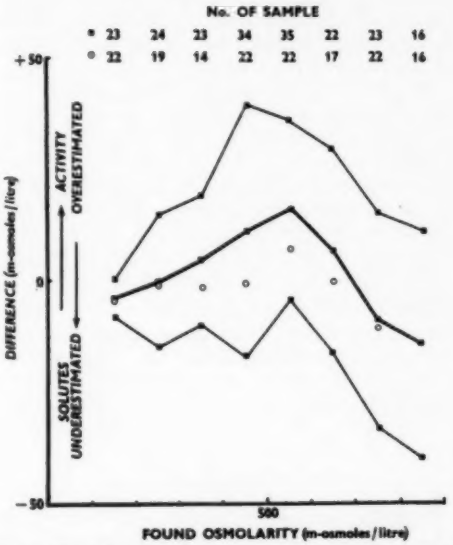

FIG. 1.—The differences between calculated and found osmolarity of urine. The calculated value is based on twice the sum of the concentrations of Na^+, K^+, and NH_4^+, and the concentrations of urea, creatinine, and protein. ■ Mean values ± standard deviation in 200 samples. O Mean values of samples when chlorothiazide had not been given.

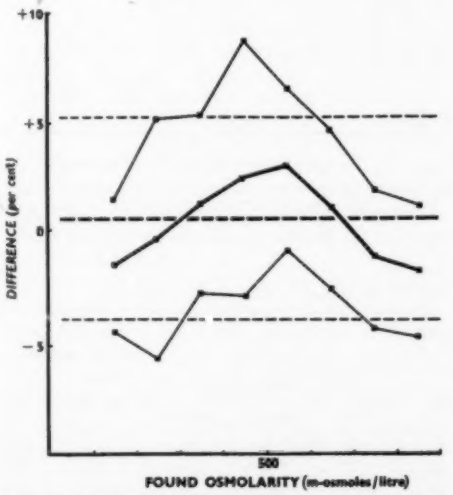

FIG. 2.—The percentage differences between calculated and found osmolarity in 200 samples of urine. Mean values and standard deviation at different osmolarities.

The percentage variations of the calculated from the found values are not large (Fig. 2). In the whole series the mean difference is small (+0.75%) and 81% of the samples fall within a 5% difference. Underestimation in the samples of lowest and highest osmolarity is of similar percentage value. Overestimation is still maximal at 500 to 600 m.osmoles/l., in some cases being more than 20%.

A high proportion of samples with overestimation were noticed to be from subjects who had had the diuretic chlorothiazide, and when these are removed from the series overestimation is only apparent in the mean figure for the one group with osmolarity of 500 to 600 m.osmoles/l. This is shown in Fig. 1. The urine after chlorothiazide tends to be alkaline, so the effect of urinary hydrogen ion concentration was studied in five subjects where variations occurred spontaneously or were induced by giving chlorothiazide or ammonium chloride. The results in the form of linear regression lines, relating the differences to *p*H, are shown in Fig. 3. In three subjects there is a marked increase in overestimation of osmolarity as the *p*H rises, in two no significant change. The increase is not related to alteration of the found osmolarity, both high and low values occurring at all *p*Hs. While these lines show the mean change with *p*H, it is not suggested that the relationship is in fact linear, and since the molar concentrations and volumes of the samples vary considerably, no quantitative relationship is apparent.

In Fig. 4 the differences in one subject in 12 consecutive half-hour specimens are shown (open circles). They vary from +31 m.osmoles/l. at *p*H 7.19 to −33 m.osmoles/l. at *p*H 5.16, the found

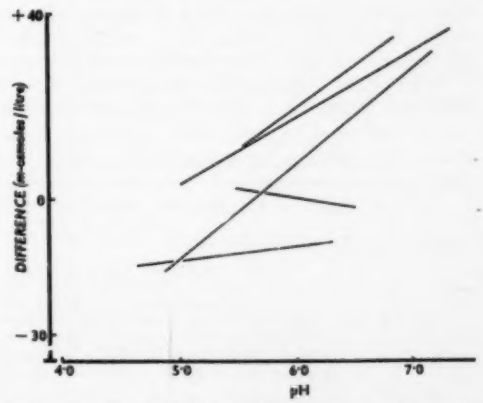

FIG. 3.—The effect of *p*H on the differences between calculated and found osmolarity in urines from five subjects, eight to twelve samples in each. The lines are linear regression equations.

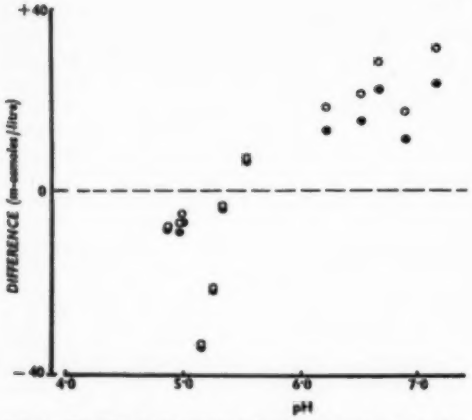

FIG. 4.—The effect of *p*H on the differences between calculated and found osmolarity in successive samples from one subject. ○ Total difference. ● Difference corrected for phosphate activity.

osmolarities in these two samples being 737 and 895 m.osmoles l. respectively. Thus the true difference between the two samples, 158 m.-osmoles/l., appears from the calculated figures to be only 94 m.osmoles/l.

The two most obvious possibilities to account for this change are that the osmotic activity of phosphate is reduced in alkaline urine owing to the increase in the proportion of HPO_4'' to H_2PO_4', and that there is less chloride, the anion with a high osmolar activity. These anions were estimated in the three experiments in which overestimation of osmolarity rose with increasing alkalinity. Some of the over-estimation is eliminated if the change in phosphate activity is allowed for. This is shown for one experiment in Fig. 4 where the closed circles show the figures corrected by subtraction of the difference between molar concentration of phosphate and its estimated osmotic activity. The mean reduction in overestimation in this experiment is 26%, in the other two with clear overestimation it is 11 and 16% respectively. No part of the change can be attributed to alterations in the Cl concentration, as the ratio Cl'/cations does not change with *p*H.

If, when chloride and phosphate are estimated, the osmolarity is calculated from these anions, the estimated cations and organic solutes, instead of assuming that the anion is chloride in amounts equivalent to the cations, the calculated values are less in all cases than the found, because the anion estimation is grossly incomplete. These osmolar differences are shown in Fig. 5 for the three experiments referred to in the last paragraph, and may be compared with those in Fig. 1. The calculated

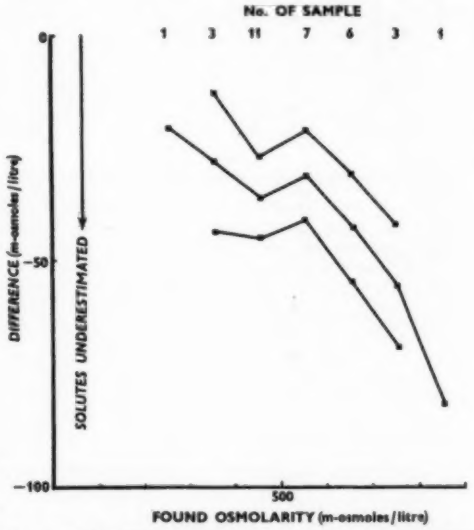

FIG. 5.—The difference between calculated and found osmolarity of urine. The calculated value is based on the concentrations of Na^+, K^+, NH_4^+, Cl', HPO_4'', H_2PO_4', urea, creatinine, and protein. Mean values ± standard deviation in 32 samples.

TABLE I

ANION AND OSMOLAR GAPS IN URINES OF DIFFERENT HYDROGEN ION CONCENTRATION

Subject	No. of Samples	*p*H Range	Cations (m.Eq./l.)	Anions (m.Eq./l.)	Anion Gap (m.Eq./l.)		Osmolarity (m.osmoles/l.)			Osmolar Gap / Anion Gap	
			Mean	Mean	Range	Mean	Found Mean	Found-Calculated Range	Found-Calculated Mean	Range	Mean
1	6	4·86–5·00	161	122	17–53	39	619	20–81	49	1·02–1·53	1·25
	6	5·55–7·19	214	159	22–94	55	569	12–70	35	0·55–0·75	0·61
2	6	5·00–5·20	167	128	27–60	40	524	24–54	33	0·52–1·15	0·86
	6	6·64–7·34	234	174	46–77	60	561	16–48	31	0·33–0·76	0·49
3	3	5·48–5·67	70	21	45–58	50	398	27–46	39	0·60–0·98	0·80
	5	6·06–6·86	89	20	58–79	69	437	31–45	39	0·55–0·73	0·57

values fall below the found by a mean of 7%, no consistent variation occurring with osmolarity. The differences are also shown in Table I, grouped according to the *p*H, together with the anion gap, or difference between estimated cations and anions, which is a measure of unestimated anions. Then the ratio osmolar gap : anion gap also given is an indication of the osmolarity of the unestimated anions, and it is seen that this is reduced in the more alkaline urines, or the osmolar activity of the unestimated anions decreases as the *p*H rises. It is thus not surprising that the calculated values exceeded the found in the more alkaline urines, when the anion was assumed to be chloride, with its high osmotic activity. The ratios are not an exact measure of the activity because cation estimation is also incomplete and the anion gap larger than that shown; they are therefore all too high. This explains the fact that the activity is in some cases apparently greater than 1.0.

Discussion

The osmolarities, calculated from the concentrations of the major cations and organic solutes, vary from the true values by less than 5% in a high proportion of the samples, and this at first sight suggests that such values can be used as reliable measurements, for variations of this order are often due to experimental error. But the fact remains that in some cases the differences are greater than 5% and that, since one factor producing wide variations has been found, the differences, at least in some cases, are true ones.

This factor is the hydrogen ion concentration of the urine. As the *p*H rises there is an increasing tendency for the calculated osmolarities to be too high, suggesting that the activity of the unestimated anions has decreased. Such a decrease is known to occur in the case of phosphate and this accounts for part of the overestimation in the experiments where phosphate is estimated, but not all.

The anion gap has been calculated in the experiments where the *p*H is known, from the difference between Na^+, K^+ and NH_4^+ concentrations and Cl' and phosphate concentrations, and compared with the osmolar gap, or difference between the found osmolar activity and the sum of the theoretical activities of all the estimated solutes. In the more alkaline urines the osmolar gap is smaller compared with the anion gap than in the more acid urines. This suggests two possibilities: that as the urine becomes more alkaline an increasing proportion of the unestimated anion is a substance of low osmotic activity, or that the activity of known or unknown solutes decreases with increasing alkalinity, possibly as a result of the formation of non-dissociating complexes. One substance of low activity which is known to increase in the urine, with alkalosis, is the trivalent ion, citrate (Evans, MacIntyre, Macpherson, and Milne, 1957), but the maximal increases in excretion recorded by these authors are less than 8 μEq./min., amounts which can only account for part of the difference between more acid and more alkaline urines found here.

The results are all given as concentrations, and calculations of the minute excretions have not been made because it is felt that, when the calculated concentrations are incorrect, multiplication by the minute volume merely adds to the complexity. The percentage differences between found and calculated osmolarities are, however, the same for excretion rates as for concentrations. In the whole series, based on anion as chloride, the mean difference between solute excretion and osmolar excretion is not significant (Fig. 2), but in the 41% of samples where there is overt underestimation of solute excretion this is 3%, measured by its osmolar activity. In the series where some anions are estimated the mean underestimation is 7%.

The most recent study of urine osmolarity is that of Yardley (1958), who made full analyses of 24-hour specimens of normal and pathological urine. The calculated osmolarities were always less than the found values by a mean of 10.6%, but anion analysis was here also shown not to be complete. This figure may be compared with the present one of 7% in the small series with separate anion analysis.

Thus it has been shown that calculation of the osmolarity from known concentrations of solutes can only give figures of uncertain value, but it should be pointed out that the reverse process of finding the solute content from the known osmolarity is even more unreliable, since the activities of the different solutes are so variable and the hydrogen ion concentration has so great an effect.

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THE URINARY EXCRETION OF 5-HYDROXYINDOLE-ACETIC ACID IN PATIENTS WITH MENOPAUSAL FLUSHING

BY

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The 24-hour urinary excretion of 5-hydroxyindoleacetic acid in women with post-menopausal flushing attacks was compared with that of a control group of post-menopausal women without flushing attacks. The results were normal in the women with post-menopausal flushing, and it is concluded that 5-hydroxytryptamine is not a factor in the pathogenesis of post-menopausal flushing.

The cause of the intermittent facial flushing which occurs frequently in some menopausal women is unknown. Flushing of the face is also a feature of metastasizing carcinoid tumours in which there is an excess of circulating 5-hydroxytryptamine, which is rapidly metabolized by monoamine oxidase to 5-hydroxyindoleacetic acid and is excreted in excess in the urine. Intravenous 5-hydroxytryptamine causes flushing and other clinical manifestations of the carcinoid syndrome (Schneckloth, Page, Del Greco, and Corcoran, 1957).

Flushing of the face is also frequent in rosacea, but there is no increase in the urinary excretion of 5-hydroxyindoleacetic acid (Rowell and Summerscales, 1960).

Materials and Methods

Twelve patients with typical menopausal flushing attacks were compared with a similar group of menopausal women who did not have flushing attacks. Details are given in Table I. The 5-hydroxyindoleacetic acid content was estimated by the method of Hanson and Serin (1955) on 4 ml. of a 24-hour specimen of urine, collected with glacial acetic acid as preservative (MacFarlane, Dalgliesh, Dutton, Lennox, Nyhus, and Smith, 1956). Readings were made on a " unicam " S.P. 600 and the results read from a calibration curve prepared from the pure substance.

TABLE I

DETAILS OF POST-MENOPAUSAL WOMEN STUDIED

	Women with Flushing Attacks	Women without Flushing Attacks
No. of cases	12	12
Age	45–55	48–58
Time since last period ..	7 months–4 years	4 months–12 years
Time since onset of flushing attacks	6 weeks–4 ,,	—

Results

These are shown in Table II. There is no significant difference in the 24-hour urinary 5-hydroxyindoleacetic acid excretion between the group of menopausal women who had flushes and those who did not ($P = < 0.9$). This confirms the observations of Munsick (1959), who found a normal excretion of 5-hydroxyindoleacetic acid in the urine of five patients who had been surgically castrated. In his patients the inhibition of flushing by the administration of oestrogens did not alter the excretion of 5-hydroxyindoleacetic acid.

TABLE II

24-HOUR URINARY EXCRETION OF 5-HYDROXYINDOLE-ACETIC ACID IN TWO SERIES OF POST-MENOPAUSAL WOMEN WITH AND WITHOUT FLUSHING ATTACKS

	Women with Flushing Attacks	Women without Flushing Attacks
Range of 24-hour urinary excretion of 5-hydroxy-indoleacetic acid (mg.)	2·1–9·8	4·2–11·5
Mean	7·63	7·65

$t = 0{\cdot}024$; $n = 22$; $P = < 0{\cdot}9$.

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THE ISOLATION AND IDENTIFICATION OF HAEMOPHILUS SPP. FROM UNUSUAL LESIONS IN CHILDREN

BY

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From a high proportion of children sent to hospital *H. influenzae* can be isolated if suitable culture media are used. A number of *H. influenzae* strains were isolated from unusual sites, such as (1) blood cultures after tonsillectomy or tonsillotomy in five cases; (2) urine or the urinary tract in eight cases; (3) the lumen of appendices removed at operation in 11 cases (4%); (4) osteomyelitis or pyarthrosis in six cases; (5) miscellaneous infections including two perianal abscesses, three cases of paronychia, one infected thyroglossal cyst, and several skin infections.

It is suggested that infections of the skeletal system and the urinary tract arise from haematogenous spread of *H. influenzae*, as demonstrated by positive blood cultures after tonsillectomy and in two cases of skeletal infection. Infection of the appendix, perianal abscesses, paronychia, and skin infections probably arise by the direct route, either by immediate contact or by passage of viable organisms through the alimentary canal.

The routine bacteriological techniques that are used in the Birmingham Children's Hospital allow *Haemophilus influenzae* to be identified in any specimen except faeces. The species of *Haemophilus* that are the subject of this communication were isolated from children. There was no opportunity to examine similar material from adults. The purpose of this paper is to draw attention to the presence of species of *Haemophilus* in sites and lesions where these organisms are not usually expected or found.

Methods and Materials

Method of Isolation.—Specimens of pus, from any site, were seeded on heated blood (chocolate) agar plates, as well as on the "composite" blood agar plate described by Rogers and Heslop (1948). Heated blood agar plates were prepared by adding 10% of oxalated horse blood to heart infusion agar at about 60° C.; after gentle mixing, the temperature was raised slowly to 75–80° C. and maintained at that temperature for 10 minutes. (The colour of the mixture should then be that of milk chocolate and there should be no gross granularity or clumping.) The molten mixture was again mixed gently and poured into Petri dishes.

All inoculated heated blood agar plates were placed in large tin containers inside which a lighted small wax candle or night light was placed to supply a higher CO_2 tension than found in normal atmosphere. The lid was sealed to the tins by a ¾ in. wide rubber elastic band. Pneumococci may require increased tension CO_2 for growth (Gladstone and Fildes, 1940; Fleming, 1941), and the presence of this gas does not impede the isolation of *H. influenzae*. This simple device was employed for every routine culture normally incubated aerobically, except those on desoxycholate citrate agar, MacConkey's, Hoyle's tellurite, or Sabouraud's media.

Early subcultures of strains of *H. influenzae* from an unusual situation were sent to Leeds for detailed identification, and typing where appropriate.

For the determination of X and V requirements, an autoclaved fresh or heated blood agar and an autoclaved yeastrel agar plate were used.

In the autoclaved blood agar plate the heat-labile V factor has been destroyed, while the heat-stable X factor is still present. Autoclaved yeastrel agar, as prepared for colony counts of water supplies (Ministry of Health, 1956), contains neither X nor V. In our experience it is the only medium consistently free from any trace of X.

Both plates were seeded evenly with the *Haemophilus* strain under test. A staphylococcus was then streaked halfway across the inoculated area. Strains dependent on X factor alone grow all over the inoculated area of the autoclaved chocolate agar plate, while on the same medium strains requiring

either V alone, or both X and V, do not grow except as satellite growth in the vicinity of the staphylococcus streak. On the yeastrel agar plate only V-dependent strains show satellitism near the staphylococcus streak. Strains requiring either X only, or both X and V, show no growth at all on yeastrel agar.

Type Identification.—For type identification, simple slide agglutination with the six type sera a to f was employed as a screening test. Floccular precipito-agglutination, similar to that seen with pneumococcus types and their sera, occurs almost instantaneously. However, a slower, granular, non-type-specific agglutination may occur with some sera and strains and is probably due to somatic antigens and their corresponding antibodies. These non-specific reactions make the slide agglutination technique less than 100% reliable, and it is therefore essential to obtain confirmation by the Neufeld technique, or, if preferred, by the formal tube agglutination method.

For the Neufeld technique, Levinthal broth cultures should not be older than five or six hours, at which time capsules are fully developed, and type-specific polysaccharides from them have not yet gone into solution in the culture medium. The age of the Levinthal broth culture can be reduced considerably if 1–2 ml. of the medium is pre-warmed to 37° C. before inoculation from an overnight chocolate or Levinthal agar plate immediately after the latter has been removed from the incubator. In this way the lag period is obviated. Capsules are well developed when the Levinthal broth culture shows the first signs of turbidity, i.e., after one or two hours if pre-warmed broth is used. Then a hanging drop preparation is made with (*a*) a loopful of the culture, (*b*) a loopful of type-specific *H. influenzae* antiserum, and (*c*) a discharged loop of Loeffler's methylene blue. The unstained capsules around the stained bacillary bodies are well defined when viewed through a microscope with corrected tube length. Cross reactions between *H. influenzae* types do not occur with the Neufeld technique or the formal tube agglutination.

Specimens.—Immediately before a tonsillectomy or tonsillotomy, a tonsillar swab and a blood culture were taken and another blood culture 3–4 min. after the operation. The blood samples were run into liquoid and were then transferred to a 0.15% glucose–1% peptone-infusion broth (Stuart, 1948). The removed tonsillar tissue was placed in a sterile Petri dish and the surface of the tissue was sterilized by searing. Then an incision was made with a sterile scalpel through the surface area thus sterilized and the fresh inner surface and depths of the crypts, exposed under sterile conditions, were cultured.

A few of the urinary strains were isolated from "clean" mid-stream specimens, but if *H. influenzae* was found in such circumstances a catheter specimen was taken, or at least a sample of urine obtained after scrupulous cleaning of the skin surrounding the meatus; from female children, a vaginal swab was taken at the same time.

Results

The strains of *H. influenzae* isolated in this way are grouped under headings according to the sites in which they were found.

Blood Cultures Immediately Before and After Removal of Tonsils.—Thirty-eight patients were examined, of whom 19 had their tonsils removed by tonsillectomy. None of the pre-operative blood cultures grew any pathogens. Five of the post-operation blood cultures grew *H. influenzae* in pure culture.

Although cultures from the tonsils usually yielded a mixed growth of pneumococci, haemolytic streptococci, and other mouth organisms besides *H. influenzae*, only *H. influenzae* was grown from the corresponding blood cultures. Four of the five strains were sent to Leeds for examination; three were not capsulated. One was a type b strain; both the pre-operative throat swab and the cultures from the tonsils of this child provided mixed growths which included non-capsulated *H. influenzae*. All colonies isolated from the tonsils and the throat of this case looked alike, but the presence of a few mucoid colonies in the cultures cannot be excluded, as a subculture from a single colony thought to be representative had been sent to Leeds for further examination.

TABLE I
ISOLATION OF ORGANISMS FROM BLOOD CULTURES AFTER TONSILLOTOMY AND TONSILLECTOMY

Operation	No. of Cases	Blood Culture					
		Pre-operative			Post-operative		
		Growth of			Growth of		
		Sterile	Coagulase-negative Staphylococci	*H. influenzae*	Sterile	Coagulase-negative Staphylococci	*H. influenzae*
Tonsillotomy	19	18	1	0	15	1	3
Tonsillectomy	19	17	2	0	16	1	2
Total	38	35	3	0	31	2	5

TABLE II

ISOLATION OF *H. INFLUENZAE* FROM TONSILS OF CASES IN WHICH THE ORGANISM WAS PRESENT IN THE BLOOD STREAM POST-OPERATIVELY

No. of Colonies of *H. influenzae* Grown	Pre-operative Tonsillar Swab	Post-operative Cultures from Tonsils
None	1	2
A few	2	2
Many	2	1

The results of the tonsillar cultures are summarized in Tables I and II. There were three positive blood cultures after tonsillotomy and two after tonsillectomy.

Urinary Cultures.—Eight children have been found to have urines infected with *H. influenzae*: none has been infected with *H. parainfluenzae*. Two of the eight children, from whom urine strains of *H. influenzae* were isolated, had a recent and violent injury. Five others had congenital abnormalities of the urinary tract. In two a calculus of the pelvis of the kidney was found and one of these two children also had hydronephrosis. *H. influenzae* was grown twice from catheter specimens and three times from clean specimens of one of these children (No. 3 in Table III).

Clinically it was noteworthy that sometimes *H. influenzae* was isolated from the urine of a child attending the hospital for the first time with what was thought to be an attack of uncomplicated pyelitis. Subsequently every one of these children returned with repeated urinary tract infections. On further investigation each of the children was found to have either renal calculi or congenital abnormalities of the urinary tract. The results are summarized in Table III.

One case deserves particular mention.

This boy had had both ureters transplanted into the colon at the age of 11 months because of a congenital ectopia vesicae. Four years later a stenosis of the right urethrocolic anastomosis developed and this was relieved by a plastic operation. Then a hydronephrosis of the right kidney developed and persisted. Six years later, at the age of 10, the boy complained of pain in the right renal region. Radiographs showed that a calculus was present in the renal pelvis. The calculus was removed to preserve the kidney and to allow the ureter to be re-implanted in the colon at a later date. When the calculus was exposed, a swab was taken from the turbid fluid in the renal pelvis. This yielded a heavy growth of type 5 pneumococci and a moderate number of *H. influenzae*. Cultures of the faeces failed to grow either of these organisms, although *E. coli*, green-forming non-haemolytic and Lancefield Group D haemolytic streptococci were isolated.

Cultures from Lumen of Appendices Removed at Operation.—For several years past, routine cultures have been made from the contents of appendices removed at operation for the purpose of identifying any *Salmonella* or *Shigella* strains (Rogers, 1957; White *et al.*, to be published). At the same time, the contents of the appendices were treated as specimens of pus even if pus was absent, in an attempt to culture pyogenic organisms.

Eleven strains (Table IV) of non-capsulated *H. influenzae* were found in 327 appendices investigated in this way (3.4%).

TABLE III

ISOLATIONS OF *H. INFLUENZAE* FROM URINARY TRACT

No.	Sex and Age	Abnormalities	Catheter Specimen	Growth of *H. influenzae*	Remarks
1	F 3	Hydroureter Hydronephrosis	No	Numerous non-capsulated *H. influenzae*, few *E. coli*	Microscopically many *H. influenzae* in urine on first attendance at hospital. Further attacks of urinary infection were due to other organisms
2	M 3	Imperforate anus; recto-vesicular fistula; permanent colostomy	Yes, through indwelling catheter	Heavy, pure	Had had many attacks of tonsillitis, very ill on admission
3	F 4	Bilateral hydroureter, hydronephrosis	Yes	Heavy, pure, non-capsulated	Grown repeatedly from catheter specimens. Patient's serum without agglutinins against own *H influenzae* strain
4	F 5	Chronic pyelonephritis; staghorn calculus	,,	Heavy, together with a few coliform bacilli and occasional *Staph. albus*	*H. influenzae* predominant
5	F 12	Bilateral hydronephrosis	,,	Heavy, pure, non-capsulated	Of many attacks of pyelitis only one due to *H. influenzae*
6	M 11	Bilateral hydronephrosis; transplanted ureters; calculus in pelvis of kidney	Direct	Heavy, non-capsulated, mixed with equal number of pneumococcus type 5 colonies	Nephrostomy specimen; for details see text
7	F 9	None; see "Remarks"	No	Moderate, together with a few coagulase-positive *Staph. aureus* colonies	Heavy 6 ft. fall 3 days before admission, heavy haematuria for 10 days; recovered when treated as pyelitis
8	F 6	Injury in kidney region	,,	Heavy, together with a few colonies of *E. coli* and *Staph. albus*	Microscopically many pus cells and *H. influenzae* in urine; developed dysuria

TABLE IV
BACTERIAL FLORA OF APPENDICES REMOVED FROM CHILDREN

Year	*E. coli**	Non-lactose Fermenting Bacilli not Salmonellae or Shigellae	Salmonellae	Shigellae	Streptococci other than β-Haemolytic	Lancefield Group F Haemolytic Streptococci	Pneumococci	*H. influenzae*	Staphylococci	No Gram-negative Bacilli Isolated	No. Appendices Cultured
1956 ..	146	44	1	12	103	21	3	2	15	13	170
1957 ..	79	14	0	2	31	14	4	2	3	4	90
1958 ..	64	10	1	0	29	11	5	7	6	3	67
1959 ..	45	44	0	2	30	5	2	4	0	2	49
Total	334	72	2	16	193	51	14	15†	24	22	376
% of total	88·8	19·1	0·5	4·3	51·3	13·6	3·7	4·0	6·4	5·9	

* One strain each of specific serological types O.125, O.119, O.111. † One strain type f.

Table IV also shows not inconsiderable yearly fluctuations of the species isolated from the lumina of appendices.

Pneumococci were present in 12 specimens (3.7%) often as the only other organism besides *H. influenzae*. The satellite phenomenon and absence of antagonism between the two organisms was noted consistently. The types of pneumococci found were 4 (1), 6 (2), 9 (3), 19 (2), 23 (2), 34 (1), and 35 (1), the figures in brackets indicating the number of strains of each type isolated.

Pus in Sites Unconnected with Respiratory, Alimentary, or Urinary Tracts.—Abscesses and osteomyelitis and pyarthrosis furnished the specimens.

Abscesses. — Non-capsulated strains of *H. influenzae* were isolated in nearly pure culture from two perianal abscesses, where they probably arrived via the gastrointestinal tract in the same way as the organism was thought to have reached the lumina of the appendices. Pure growth of *H. influenzae* was obtained from pus of an infected thyroglossal cyst.

A comparatively rare species of the genus *Haemophilus, H. parahaemolyticus,* which is sometimes found in acute tonsillitis and subacute endocarditis (Pittman, 1953), was isolated, together with coagulase-positive *Staphylococcus aureus* and *albus,* from an abscess under the left mandible in a boy aged 15 months. The dental surgeon who incised the abscess stated: "The infection obviously comes from the mandible as bare bone was easily palpable through the incision."

In 1929, Fleming reported the isolation of *H. influenzae* from septic wounds. Similarly we succeeded on several occasions in its isolation in mixed culture from infected skin, also from the pus of paronychiae. One was from the finger of a boy aged 6 years, when non-capsulated *H. influenzae* was present together with *Staphylococcus aureus* and *Strep. pyogenes.* In another similar case, a boy of 4 years, non-capsulated *H. influenzae* was isolated together with haemolytic streptococci of Lancefield's group F, which normally is a commensal in the nasopharynx (Long and Bliss, 1934; Hare and Maxted, 1935). It is interesting to note that both these boys were finger suckers. A third paronychia yielded a mixed culture of *Staphylococcus aureus* and a non-capsulated strain of *H. influenzae.*

Osteomyelitis and Pyarthrosis.—There were three cases of osteomyelitis of long bones, one with osteomyelitis of the tibial end of the knee and two with pyarthrosis infection in the hip joint, and also one child with osteomyelitis of the os calcis. Two children had an acute pyarthrosis of the hip, in all six cases with involvement of bones or joints. One of the cases of pyarthrosis of the hip was caused by a non-capsulated strain of *H. influenzae*: and a second non-capsulated strain was isolated from a case of osteomyelitis of long bone, but in this case the presence of a capsule initially cannot be ruled out because, owing to the long holiday period, the strain was subcultured for several weeks before it could be typed. From all other cases, *H. influenzae* type b was isolated, and this type was also recovered from the blood cultures of two of the children, one with pyarthrosis of the hip, the other with osteomyelitis of the tibial end of the knee joint.

Discussion

From these observations the question arises how *H. influenzae,* which is a pathogenic organism of the upper, and sometimes the lower, respiratory tract, could establish itself in the situations from which it was isolated. The literature on *H. influenzae* meningitis and epiglottitis makes it clear

that invasion of the blood stream by *H. influenzae* is not such a rare occurrence as one might be led to believe (Zinnemann, 1960). Once *H. influenzae* has been carried by the blood stream to a tissue with lowered local resistance, establishment of a focus of infection is a possible consequence.

Thus the strains of *H. influenzae* isolated from urinary tract infections in children with either congenital abnormalities of the urogenital system or with stone formation in the pelvis of the kidney cannot have reached these loci minoris resistentiae except as a reult of a transient bacteraemia. The work of Rocha, Guze, Freedman, and Beeson (1958) and Freedman and Beeson (1958) offers an explanation of unusual susceptibility of the kidney to infection and in particular of the increased vulnerability of damaged kidneys. They showed that when the renal medulla was damaged the nephrons draining through that part of the kidney were particularly susceptible to a pyogenic infection if a bacteraemia recurred. Albright, Dienes, and Sulkowitch (1938) also reported repeated isolation of *H. influenzae* from the urine of two adults suffering from pyelonephritis and nephrocalcinosis. Thus the identification of *H. influenzae* in an infected urine indicates the need for detailed investigation of the patient to exclude the presence of abnormalities. The case in which turbid fluid obtained at nephrostomy contained non-capsulated *H. influenzae* and a pneumococcus type 5 emphasizes the pathogenesis of such infections of the urinary tracts, because there can be no doubt that this specimen came from the kidney.

One of the ways in which such infections can start has been demonstrated by the finding of *H. influenzae* in blood cultures after removal of the tonsils. The isolation of *H. influenzae* from blood cultures taken after such operations was somewhat unexpected, because Okell and Elliott (1935) never isolated *H. influenzae* in any of their positive blood cultures before or after tooth extractions, although they isolated more than one organism on several occasions, and Ross and Rogers (1943) isolated streptococci but not *H. influenzae* after root canal instrumentation of infected but viable teeth. Our findings were all the more surprising because a selective bacteraemia must have taken place, for on several occasions only a few colonies of *H. influenzae* were present in either pre- or post-operative tonsillar swabs together with other more numerous micro-organisms. However, *H. influenzae* could always be isolated from either the pre- or post-operative tonsillar swab or culture when it was found in the blood stream after tonsillectomy. We know only one report in the literature that bears on this point, a case reported by Clarke (1912), of a girl aged 13 with chronic, intermittent otitis media due to *H. influenzae*, who developed septicaemia and osteomyelitis following tonsillectomy.

The case of Clarke also demonstrated for the first time that infection of the skeletal system with *H. influenzae* can be traced back to bacteraemia. There are only a few references in the literature to osteomyelitis and pyarthrosis due to *H. influenzae*. The 31 cases reported between 1899 and 1938 have been reviewed by Weaver and Sherwood (1938), who added two cases of their own. Both these, one an adult coloured woman and one a coloured child of 18 months, had pyarthrosis. In 1947, Hirsch and Nassau described one case with osteomyelitis and abscesses of the subcutaneous tissue; this was due to untyped *H. influenzae* and followed an acute febrile pharyngitis within four days. In the era before the advent of antibacterial drugs Weaver and Sherwood (1938) could point out that when osteomyelitis or pyarthrosis was preceded, accompanied, or followed by meningitis due to *H. influenzae* survival could not be expected, whilst practically all cases suffering only from *H. influenzae* infection of the skeletal system recovered, usually with a high degree of mobility. Antibiotics and sulphonamides have changed the prognosis of meningitis due to *H. influenzae* so much that nowadays a case fatality rate of more than 4% is regarded as a reason for a review of therapeutic procedures (Zinnemann, 1960).

It is suggested that a different mechanism operates when *H. influenzae* establishes itself as the cause of infection in an appendix or causes a perianal abscess. Although infection of the blood stream cannot be ruled out, the direct route via the upper respiratory and the alimentary tracts seems more plausible, particularly if one assumes that micro-organisms enveloped in a ball of highly viscous mucus or mucopus, which is a characteristic of *H. influenzae* infections, may pass the zones of gastric and duodenal digestion unharmed. Fleming (1929) previously isolated *H. influenzae* from the faeces. Two groups of Australian workers have published evidence to support their contention that oropharyngeal secretions reached the intestines. Anderson and Langford (1958) isolated three strains of *H. parainfluenzae* out of 12 jejunal specimens aspirated from normal children and three from 15 children with coeliac disease. Bishop and Allcock (1960) examined fluid aspirated from the small intestine above obstructions; from 27 patients they isolated two strains of *H. parainfluenzae* and *H. aphrophilus*. Neither of these groups isolated any strain of

H. influenzae or *H. parahaemolyticus.* Hare and Maxted (1935) recovered *Streptococcus pyogenes* from the faeces of cases of scarlet fever, which findings tend to support the assumption of respiratory organisms passing through the alimentary tract in a viable state.

The isolation of *H. influenzae* from a skin lesion was reported by Fleming in 1929; he used penicillin incorporated in culture media for its isolation. Penicillin was not a constituent of the routine nutrient media used in this investigation, and it is possible that some strains from this site have been missed. On the special blood agar plates used (Rogers and Heslop, 1948), the solution of 1 in 10,000 gentian violet, which is spread over the outer segments, nearly always inhibits *H. influenzae.* This inhibition is helpful in the identification of *H. influenzae.* Inhibition of *H. influenzae* also takes place at a 50 times higher dilution of gentian violet, which is the concentration of the dye in Garrod's blood agar plate (1942). These observations are not in accord with the findings of Bernstein and Loewe (1919) or of Masters, Brumfitt, Mendez, and Likar (1958), who used gentian violet in solid media to facilitate the isolation of *H. influenzae.*

It is important that the isolation of *H. influenzae* in unusual sites should be seen in perspective. For this reason a survey of the number of strains isolated from other routine specimens during 1957 at the Children's Hospital is given in Table V.

TABLE V

SURVEY OF NUMBER AND PERCENTAGE OF STRAINS OF *H. INFLUENZAE* ISOLATED FROM ROUTINE SPECIMENS DURING 1957 AT THE CHILDREN'S HOSPITAL, BIRMINGHAM

Type of Examination	Total Specimens	*H. influenzae* Present		*H. influenzae* Predominant Organisms		
		No. Isolated	%	No. Isolated	% of Total Isolations	% of All Specimens
Throat	863	389	45	76	20	9
Nose	136	71	52	36	50	22
Ear	238	63	26	25	40	1
Conjunctiva ..	61	24	39	11	46	18
Antra	72	35	48	16	46	22
Cerebrospinal fluid	24	10	42	10	100	42

Only the 10 strains of *H. influenzae* isolated from cerebrospinal fluid were typed; all were of type b. The other 582 strains were not so examined. These figures are presented to show how frequently *H. influenzae* is found in children attending hospital.

If routine cultures are made with a technique that permits the growth of *H. influenzae,* then the results obtained in Birmingham and reported here probably would be a common experience. In this sense we do not regard our findings as exceptional.

We are pleased to acknowledge the help of Dr. J. Boissard, who confirmed the identity of the early strains of Group F haemolytic streptococci; to Drs. G. M. Williamson and Ann W. Cooper for identifying some strains when one of us was absent; to Mr. Guy Baines, who has allowed us to report the child from whom the nephrostomy specimen was obtained; to Mr. G. S. Hoggins for permission to describe the case infected with *H. parahaemolyticus*; and to Mrs. D. Clarke for technical assistance.

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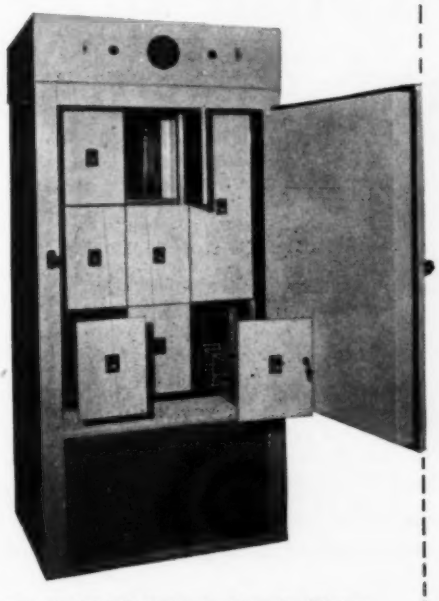

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J. clin. Path. (1960), **13**, 525.

TECHNICAL METHODS

A Simple and Rapid Technique for the Identification of Proteus-Providence Strains

U. BACHRACH

From the Department of Clinical Microbiology, Hebrew University—Hadassah Medical School, Jerusalem, Israel

(RECEIVED FOR PUBLICATION JUNE 3, 1960)

In a previous communication it was proposed to use a filter paper strip impregnated with urea for the detection of urease activity of *Proteus* strains (Bachrach and Wormser, 1959). Urease activity is not limited to *Proteus* strains, for other bacteria, such as *Corynebacterium pseudodiphtheriticum* (cf. Breed, Murray, and Smith, 1957) and some *Klebsiella* strains, are also active (Epstein, 1959). On the other hand, Providence strains, which are included in the genus *Proteus*, are urease negative (Shaw and Clarke, 1955). The urease test is therefore not a reliable method for the identification of bacteria belonging to the Proteus–Providence group.

An additional test has been added to our filter paper strip technique to make it a reliable method for the identification of bacteria belonging to the Proteus–Providence group. The additional test is based on the well-known fact that these strains rapidly deaminate tryptophan to indolepyruvic acid, which can be detected by ferric chloride (Singer and Volcani, 1955; Falkow, 1957).

Reagents

Impregnation Solution.—Dissolve 1.0 g. urea, 0.1 g. l-tryptophan, and 0.1 g. phenolphthalein in 10 ml. 96% ethyl alcohol.

Saline Solution.—Make up 8.5 g. sodium chloride in 100 ml. distilled water.

Dilute Hydrochloric Acid Solution.—Add 1 ml. concentrated hydrochloric acid to 100 ml. distilled water.

Sodium Hydroxide Solution.—Add 0.4 g. sodium hydroxide to 100 ml. distilled water.

Hydrochloric Acid Solution (50%).—Add 50 ml. concentrated hydrochloric acid to 50 ml. distilled water.

Ferric Chloride Solution.—Dissolve 10 g. ferric chloride in 100 ml. distilled water.

Technique

Sodium hydroxide solution is added drop by drop to the impregnation solution until the indicator turns pink. Diluted hydrochloric acid is then added till the solution becomes just colourless. Whatman No. 3 filter paper is soaked with the colourless impregnation solution, dried at room temperature, and cut into strips (15 by 5 mm.) which are kept in a dry test-tube.

The bacterial strain to be tested is grown on a liquid or solid medium. If a liquid medium is used, the cells are harvested by centrifugation (2,500 r.p.m.) and the supernatant discarded. Bacteria grown on solid media are scraped off with a bacteriological needle and suspended in 0.1 ml. saline solution in a small test-tube (9 by 100 mm.). After immersing the impregnated filter paper strip in the bacterial suspension, the test-tube is placed in a 37° C. water-bath and examined after one to two hours. A red coloration of the filter paper strip indicates the presence of urease in the bacterial cells.

The presence of tryptophan deaminase activity is demonstrated by the addition of one drop of the 50% hydrochloric acid solution and one drop of the ferric chloride solution to the filter paper still immersed in the bacterial suspension. A brown colour appears within two minutes, when the result is positive.

Results

Various *Enterobacteriaceae* were examined by our modified filter paper strip technique. Only *Proteus* and Providence strains deaminated tryptophan (Table I). Other bacteria, such as *Shigellae*, *Salmonellae*, *Escherichiae*, and *Klebsiellae*, gave negative results. As was to be expected, *Proteus* strains were urease positive when tested by the filter paper strip technique, whereas bacteria of the Providence group were negative. One of the *Klebsiella* strains examined was urease positive (Table I).

TABLE I

BEHAVIOUR OF VARIOUS BACTERIA EXAMINED BY FILTER PAPER STRIP TECHNIQUE

Organism	No. of Strains	Filter Paper Test			
		Urease		Tryptophan Deaminase	
		Positive	Negative	Positive	Negative
Proteus	32	32		32	
Providence ..	20		20	20	
Salmonella	23		23		23
Shigella	22		22		22
Escherichia ..	20		20		20
Klebsiella	5	1	4		5
Bethesda-Ballerup	13		13		13

Discussion

The development of a technique demonstrating both tryptophan deaminase and urease activities in one test is thus of great help in the identification of Proteus–Providence strains.

As has already been pointed out, bacteria grown on various culture media may be employed in the filter paper strip test, which has the advantage of being simple and rapid (Bachrach and Wormser, 1959). The test is very sensitive, and pure culture is not required. Furthermore, the filter paper strip, which is easily prepared at low cost, may be kept for a long period without deterioration.

The use of filter papers for the identification of bacteria has already been suggested by different authors. Knox (1949) placed filter paper strips impregnated with carbohydrates on inoculated agar plates and noted the subsequent change of the indicator. Paper discs containing culture media were employed by Snyder (1954) for the differentiation of bacteria. Similar techniques were described by Sanders, Faber, and Cook (1957) and Cook and Pelczar (1958).

Summary

A filter paper strip impregnated with urea, phenolphthalein, and tryptophan was used in the identification of bacteria of the Proteus–Providence group.

Bacterial cells were suspended in 0.1 ml. saline, and the impregnated filter paper strip inserted into the suspension. After one to two hours of incubation at 37° C., urease activity was detected by a change of the colour of the indicator to pink.

Tryptophan deaminase activity was demonstrated by the addition of one drop of hydrochloric acid and ferric chloride solutions. A brown coloration indicated a positive reaction.

Of all *Enterobacteriaceae* tested, only Proteus–Providence strains gave positive results.

I am deeply grateful to Mr. M. Wormser for valuable technical assistance.

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A New Method of Demonstrating Capsulated *Bacillus anthracis*

M. A. SOLTYS

From the Department of Animal Pathology, University of Cambridge

(RECEIVED FOR PUBLICATION AUGUST 10, 1960)

In order to show the capsules of anthrax bacilli, Heim (1901) and M'Fadyean (1903) stained them by 1% aqueous solution of methylene blue. According to M'Fadyean (1903, 1909) films made from blood or tissue of an animal which died from anthrax should be gently heated, or preferably only dried in order not to destroy the capsule. Such films, stained by 1% methylene blue for about three to five minutes and examined microscopically, showed blue-stained bacilli, surrounded by a deposit of granular or amorphous material scattered among the bacilli coloured reddish purple, representing the debris of imperfectly fixed capsules.

The slight fixation by heat results of course in a danger of infection from the slide. Despite the precautions normally taken to prevent infection with *Bacillus anthracis*, a laboratory worker sometimes becomes infected from slides. Soltys (1948) reported anthrax in a laboratory worker infected from a stained film. He showed that ordinary fixation and staining of films of anthrax bacilli have no bactericidal effect on anthrax bacilli. Even films containing non-sporulating forms, made from the infected tissue, after being only gently heated to preserve the capsule are still dangerous, as such films left for some time will sporulate.

In order to kill the organism, but at the same time to preserve the capsule, various chemicals which kill spores of *B. anthracis* were tested. Of all chemical compounds tested, Zenker's solution used for the fixation of tissue for histological studies provided the best results. It was found that once the preparation was fixed with Zenker's solution it could be stained by any available stain at any time. The organism takes on the colour of the stain, while the capsule remains as a well-defined clear zone. If films fixed by Zenker's solution are stained by methylene blue, the capsule is stained faintly purple. Films should be washed with water after being fixed by Zenker's solution. This method has been used by the writer since 1948 in his classes for students and also for routine diagnostic work, and has proved to be far superior to the old method recommended by M'Fadyean. In addition to being a much better method it is completely safe, as anthrax bacilli are killed.

In order to show that slides containing both capsulated bacilli and sporulating bacilli were saf

they were washed with wet swabs and suspended in 1 ml. of nutrient broth and inoculated on blood agar media and into mice. All tested slides failed to produce either culture or infection.

A similar experiment was performed with the same type of slides from which mercuric chloride was removed. Both the medium and mice failed to show the presence of *B. anthracis*.

Because unstained areas round the bacteria may simply be the space between a lightly stained surrounding medium, which retracted from the cells on drying and bacteria, the best way to demonstrate capsules is actually to stain them by some procedure which differentiates them from the cell itself. A modification of Leifson's method (1930) for staining flagella accomplishes this in the case of *B. anthracis*.

Staining Procedure

Fix the films in Zenker's fluid for one to two minutes and wash in water. Then flood slides with the solution A for 10 minutes. Wash with tap water and stain with solution B for five to 10 minutes. Wash in tap water, dry, and examine.

Solution A

$NH_4Al(SO_4)_2 12H_2O$ (sat. aqueous solution) ..	20 ml.
Tannic acid (20% aqueous solution)	10 ,,
Distilled water	10 ,,
Ethyl alcohol 95%	15 ,,
Basic fuchsin (sat. solution in 95% ethyl alcohol)	3 ,,

The stain deteriorates after a week and should be freshly prepared.

Solution B

Methylene blue in powder	0·1 g.
Borax	1 ,,
Distilled water	100 ml.

Anthrax bacilli stained by the second method appear as dark blue bacilli surrounded by pale blue capsules. Background and red cells are stained red.

The first method (films are fixed in Zenker's solution for three to five minutes, washed with water, stained with methylene blue, and washed in water) is recommended for routine diagnostic work in the field by a veterinary surgeon or medical practitioner, but the second method by which capsules as well as bacteria are stained is the better for laboratory and teaching purposes.

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A Simplified Method for the Quantitative Bacterial Culture of Urine

D. J. O'SULLIVAN, M. G. FITZGERALD, M. J. MEYNELL, AND J. M. MALINS

From the General Hospital, Birmingham

(RECEIVED FOR PUBLICATION JULY 22, 1960)

The value of quantitative bacterial culture of freshly voided clean mid-stream urine in assessing infection of the urinary tract is established (Kass, 1955; Boshell and Sanford, 1958; Monzon, Ory, Dobson, Carter, and Yow, 1958). However, the technique of serial dilution recommended is time consuming and requires a large number of petri dishes and pipettes, so that its use on a large scale in a clinical laboratory is impractical. It was therefore decided to compare the colony counts obtained by the dilution technique with those obtained by culture of a "standard" loopful of urine. The results with the latter are sufficiently accurate for the purpose of assessing urinary tract infection in clinical practice.

Collection of Specimens

The urine was collected in the course of an investigation into the prevalence of urinary tract infection in diabetic and general populations. There were 100 urines in all, from 39 men and 61 women. A high proportion of the subjects were middle-aged and elderly.

In males the glans penis was swabbed with 1:1,000 benzalkonium hydrochloride ("roccal") and a mid-stream specimen of urine collected. Female patients were prepared in the dorsal lithotomy position as for catheterization with the same antiseptic. The labia were separated, the patient was then encouraged to void urine and the specimen was collected from the mid portion of the urinary stream into a sterile container. Specimens were dealt with immediately after collection; if this was not possible they were stored at −20° C.

Method

The number of viable organisms per millilitre of urine was determined by the same observer with each specimen of urine as follows:

Dilution Technique.—Serial tenfold dilutions of urine from 1 in 10 to 1 in 1,000,000 were made in sterile normal saline and 1 ml. of each dilution mixed with 10 ml. of molten nutrient agar kept at 50° C., then poured into a petri dish and allowed to set. The six plates were incubated aerobically at 37° C. for 48 hours. The plate containing approximately 50–200 colonies was counted, and the result multiplied by the dilution gave the number of viable organisms per ml.

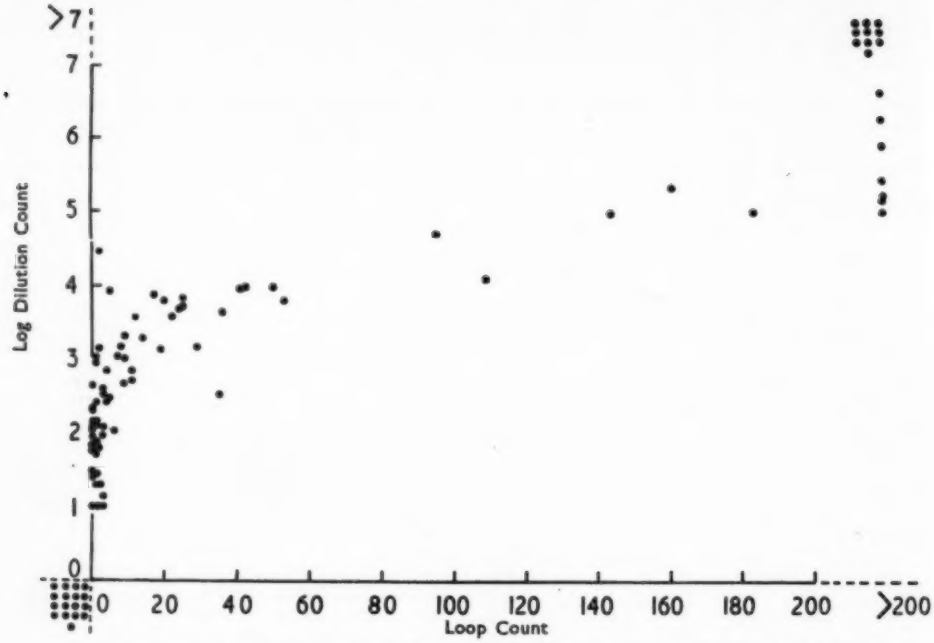

FIG. 1.—Log dilution loop count plotted against actual loop count from the same specimen (100 mid-stream urines).

Standard Loop Technique.—One loopful of undiluted freshly voided urine was spread over a nutrient agar plate. The loop (platinum wire 22 standard gauge, 3 mm. internal diameter) was removed vertically from the sample and both sides rubbed over the surface of the medium, taking care not to reinfect the loop with the original inoculum. After 48 hours' aerobic incubation at 37° C. the colonies were counted. Anaerobic culture on blood agar was performed in the early stage of the investigation. No anaerobes were isolated and this was discontinued.

Results

The results are shown in Fig. 1, in which the log of the bacterial count by the dilution method is plotted against the actual counts obtained by the loop method. Seventeen of the urine specimens cultured by the dilution method gave bacterial counts in excess of 100,000 organisms/ml. and in 16 of these the corresponding loop count was in excess of 200. The one exception, a count of 191,000 organisms by the dilution method, grew 161 colonies by the loop method.

Six counts by the dilution method were in the range of 10,000 to 100,000 organisms/ml. In five of these the corresponding loop count was between 95 and 185. In one, a dilution count of 31,000/ml. grew only two colonies by the loop method. All counts of less than 1,000 organisms/ml. by the dilution method yielded counts of less than 60 by the loop method.

All authors are agreed that dilution counts of over 100,000 organisms/ml. on voided mid-stream urine are indicative of urinary tract infection. The significance of counts between 10,000 and 100,000 organisms/ml. is less certain (Kass, 1956; Huvos and Rocha, 1959), and they should be repeated. These results show that, using the loop method, colony counts of more than 200 are indicative of urinary tract infection, counts between 90 and 200 are in the doubtful range and should be repeated, and all counts below 90 are probably the result of contamination.

Summary

The results of enumeration of viable organisms in freshly voided urine were compared by counting the colonies after serial dilution and by spreading a standard loopful of the same urine over the surface of an agar plate. The simpler loop technique appears to be sufficiently accurate for clinical purposes.

We wish to thank Miss M. A. Butcher for technical assistance.

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On Determining the Packed Cell Volume

H. E. HUTCHISON

From the Department of Haematology, University and Western Infirmary, Glasgow

(RECEIVED FOR PUBLICATION APRIL 30, 1960)

The technical simplicity of estimating the packed cell volume seems to arouse a false sense of security in its reproducibility. It is, for example, generally held by clinicians to be less subject to technical artefact than measurement of Hb concentration. Yet it has been experience here that, when the M.C.H.C. of the same patient has been determined two or three times a week over a month or so and fluctuations have occurred, they have been due to a variation in the figure recorded for the P.C.V. and not in the Hb concentration. Since becoming conscious of the possibility of significant errors in P.C.V. determination as ordinarily carried out, two main sources of error have been found, namely, errors in reading the haematocrit tube, and errors through failing to achieve uniform and adequate packing of the red cells.

In the actual reading of the haematocrit tube, two important points emerged after watching several technicians reading the same tube. First, there were variations in the angle (to the vertical) at which the tube was held; these were overcome by using the Wintrobe stand to keep the haematocrit tube vertical. Secondly, the angle to the horizontal at which the reader observed the top of the packed cell layer varied. The ideal position is to have the reader's eye at the same level as the top of the packed cell layer. Magnification, by enabling the reader to line up the back with the front of the buffy coat when taking the reading, ensures a close approximation to the ideal level. In addition, magnification allows evaluation of the level of the packed cell layer to within half or quarter of a division; it also becomes possible to make an approximate allowance in admixture of red with the white cells, when, as sometimes happens, separation of the two is not sharp. Magnification therefore adds precision to the reading.

Failure to achieve uniform and adequate packing of the red cells is the second important source of error, and may result from use of several different centrifuges for P.C.V. determination in the same laboratory, for they are not equally effective in producing the necessary centrifugal force. Even a centrifuge designed expressly for the determination of the P.C.V. may be fundamentally unsuitable for the purpose and, as an illustration of this, experience in this laboratory with the M.S.E. haematocrit centrifuge is revealing.

According to Wintrobe (1956), 30 minutes at a force of 2,264 G is required to produce so-called "complete" packing, i.e., more time at this force will not produce further packing. The M.S.E. haematocrit centrifuge at its governed speed of 3,000 r.p.m. exerts only an R.C.F. of 1,360 G, for the radius of the centrifuge is only 13.5 cm., and to produce 2,264 G a speed of 3,800 r.p.m. would be required. The machine cannot reach this speed even following maximum adjustment by the works engineer. The maximum speed is 3,375 revs. and at this it develops an R.C.F. of only 1,780 G, which is still far short of the 2,264 G specified by Wintrobe. In brief, the centrifuge has been wrongly designed. The radius is too short, being only 13.5 cm. instead of 22.5 cm.

If these conclusions regarding the M.S.E. haematocrit centrifuge are correct, packing should be incomplete at 30 min., the period recommended by the manufacturers, and this proves to be so. Thus, if after reading the P.C.V. the tubes are respun for a further 30 min. a significant increase in packing is obtained, especially obvious, of course, with blood samples in which the P.C.V. is not greatly reduced. Secondly, since packing is incomplete, the M.C.H.C. level should tend to be generally a little lower than the normal 32–36%, particularly when normal range Hb concentrations are considered, as these will have a P.C.V. in the 40% range. What is found is as shown in the table.

M.C.H.C. IN 50 BLOOD SAMPLES WITH Hb CONCENTRATION OF 96% HALDANE OR OVER

M.C.H.C.	% of Cases
Less than 31	12
31	32
32	38
33	14
34	2
More than 34	2

Comment

Less care than is required is usually given to reading the P.C.V. tubes. Indeed, the subject is not mentioned in any detail in most standard texts as being worthy of special consideration, and experience here illustrates the fallacy in this common attitude. Nor is it customary to consider the force applied by the centrifuge in G. Instead, the figure of 3,000 r.p.m. for 30 min. is too often all that is thought important. That this should occur even in the case of a centrifuge designed expressly for determining the P.C.V. is an indication of how widespread this erroneous impression may be.

I am indebted to Dr. A. Thom, Department of Engineering, University of Glasgow, for his surveillance of the R.C.F. calculations, etc., quoted.

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Dark-field Illumination for Photographing Precipitin Bands in Gel

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(RECEIVED FOR PUBLICATION AUGUST 3, 1960)

A variety of techniques for photographing precipitin bands in gel have been published, some using forms of dark-field illumination (Hunter, 1959), others using bright-field illumination (Dike, 1960). Dark-field lighting gives better visual contrast with faint unstained bands and has also been favoured for photographic work. Jackson (1959) compared various lighting systems and photographic techniques and found that, with unstained preparations, a dark-field method gave better results. With stained bands, the relative efficiency of the lighting methods was influenced by the colour of the stain employed; with an orange stain, dark-field lighting gave better results, with a blue stain good results were obtained by either method.

The influence of the angle of illumination on photographic contrast has been investigated by Hunter. He adopted a bilaterally symmetrical dark-field lighting system in which the Petri dish was illuminated from below by two 500 w. spotlights with their beams converging in a vertical plane and passing through the agar at 21° to the normal. With complex serological systems, Hunter found that greatest separation of bands was obtained when they lay parallel to the light plane. It follows that uniform separation of bands in asymmetrical patterns cannot be achieved by this method. In comparative studies, where it is important to ensure uniform separation of bands and to record faithfully their relative density, radially symmetrical lighting would appear preferable.

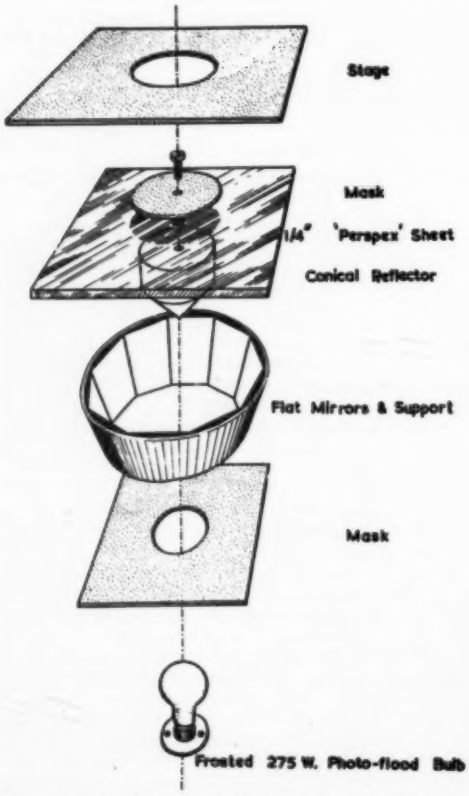

FIG. 1.—"Exploded" perspective drawing of the main components.

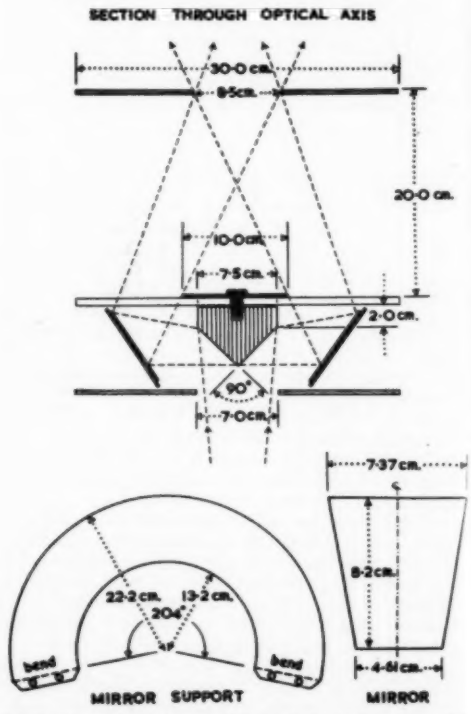

FIG. 2.—Vertical section through the optical axis showing the light path and dimensions of the main components (not to the same scale).

Ouchterlony (1958), Schutz (1958), and Reed (1960) have described radially symmetrical dark-field systems in which a ring-shaped lamp, or a masked *x*-ray viewing box, was used as a light source. An alternative method, which is both versatile and inexpensive, has been used routinely in this department for the past year. The equipment is designed to give uniform dark-field illumination at 21° to the normal for Petri dishes up to 9 cm. in diameter. Only one light source is required. Electronic flash or a variety of tungsten lamps can be interchanged readily where use of a variety of photographic emulsions makes this desirable.

The main components of the apparatus are illustrated in Fig. 1, and the light path and dimensions are shown in Fig. 2.

The conical reflector was turned from 3 in. brass rod, the base drilled and tapped, and the reflecting surface carefully polished to remove all trace of tool marks. The 10 flat reflectors were cut from 3¼ by 3¼ in. lantern slide cover glasses. The conical and flat reflecting surfaces were then coated with aluminium in a high vacuum evaporation unit of the kind used in conjunction with an electron microscope. (Where this equipment is not available, several alternatives can be adopted. The conical reflector can be chromium-plated or turned from any colourless metal which will take a high polish and, where necessary, the surface protected with a thin coat of colourless varnish. The flat reflectors can be silvered or cut from thin glass mirror.)

The supporting collar for the flat reflectors was cut from 18 gauge sheet metal, and the ends were drilled and bent upwards at right angles. The arc was then bent to form a truncated cone, and the ends bolted together.

The flat reflectors were mounted by placing them face downwards in a flat arc with their adjacent edges parallel and slightly apart. These edges were bound together by strips of adhesive tape, and then the two ends of the arc were brought together and joined in the same fashion.

The stage and masks were painted matt black on both sides, and the various components were mounted coaxially in a suitable framework. A wall-mounted enlarger fitted with a copying head and a 13.5 cm. lens was used as the camera; it was therefore convenient to arrange the stage at bench level. With a 275 w. photoflood bulb mounted at floor level, uniform exposures of 10 sec. at *f*/8 were required for Ilford G.50 ortho half-tone backed plates with normal development in ID 13 developer.

Before photographing precipitin bands the agar was carefully washed and covered with distilled water to prevent undesirable reflections from the sides of the wells. Scratches on the underside of the Petri dish were hidden by mounting the dish in a small pool of glycerol on a sheet of optically flat glass.

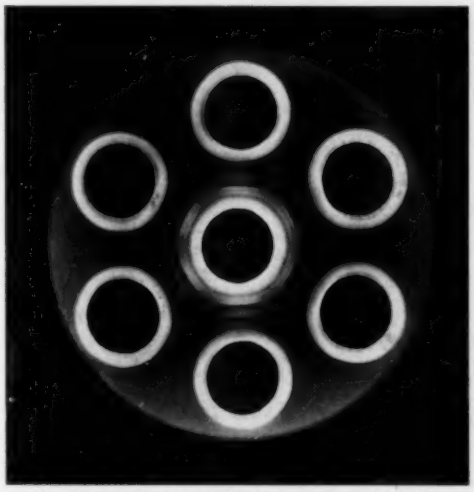

FIG. 3.—Photograph of a radially symmetrical serological system showing uniform lighting of precipitin bands. The six peripheral wells were filled with sonic-disintegrated *Leptospira icterohaemorrhagiae*; the central well contained the homologous antiserum.

Where photographs of complex serological systems containing faint but adequately resolved bands are intended for publication it is suggested that the print should be prepared from a reversed negative. With such a photograph—showing dark precipitin bands against a pale background—there is less chance of delicate bands being obscured by ink spreading in the final illustration. Cross-printing of negatives also offers further opportunity for controlling contrast, although negatives prepared with the apparatus described here printed satisfactorily on normal grades of photographic paper.

The equipment was constructed in the course of studies financed in part by a New Zealand national research fellowship. I am indebted to Mr. G. A. Wilson for technical assistance.

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A Simple Method of Photographing Ouchterlony Plates

J. H. JONES AND R. J. MARSHALL

From the Institute of Pathology and the Department of Medical Photography, Royal Infirmary, Cardiff

The Ouchterlony technique of demonstrating precipitin bands in an agar gel (Ouchterlony, 1948) is one which is now widely used for diagnostic purposes. The difficulties involved in making a permanent accurate record of the results, an essential part of the technique, are indicated by the variety of photographic methods that have been used, the majority of which are time-consuming and involve the use of special photographic equipment.

We have been using a modification of a method first described by Bartlett (quoted by Gibson, 1954) which is accurate, rapid, and requires a minimum of equipment. This technique consists essentially of making a photogram of the agar plate on a lantern plate. Because of its simplicity it is of value to those using the gel-diffusion technique.

Material and Methods

Agar Plates.—The 1% agar used is prepared according to the method of Feinberg (1956). The petri dishes are selected free from scratches and other defects in the glass. Before photographing it is an advantage to wash the surface of the agar several times with cold water to free it of dust particles. The surface of the agar is then covered with a layer of water which serves both to fill the cups, thus making them less prominent on the finished photograph, and to obliterate any surface defects of the agar.

Photography.—The lantern plates used are Ilford "special contrasty" grade. One is placed under the petri dish and exposed to light from a condenser enlarger. Bartlett's original method specified the use of a cold-cathode enlarger, though any vertical enlarger may be used; in fact, any point source of light is satisfactory. The illumination provided by an ordinary 60-watt pearl electric light bulb enclosed in a metal can having a ¼ in. diameter circular hole on its base has been used here. The lamp should be placed several feet above the precise centre of the plate.

Exposures, using an ordinary enlarger with its lens stopped down to an aperture of *f*/22 or a screened pearl bulb, are in the range of seven to 10 seconds. Processing should be in accordance with the manufacturer's instructions.

This method produces a very satisfactory negative recording faithfully all the bands in the agar plate. From this contact prints or enlargements may be made. An even simpler method is to make the photogram direct on to contrasty bromide printing paper (Ilford grade 3 or 4) though, of course, a negative image of the bands is produced, but this appears to be quite satisfactory for routine purposes.

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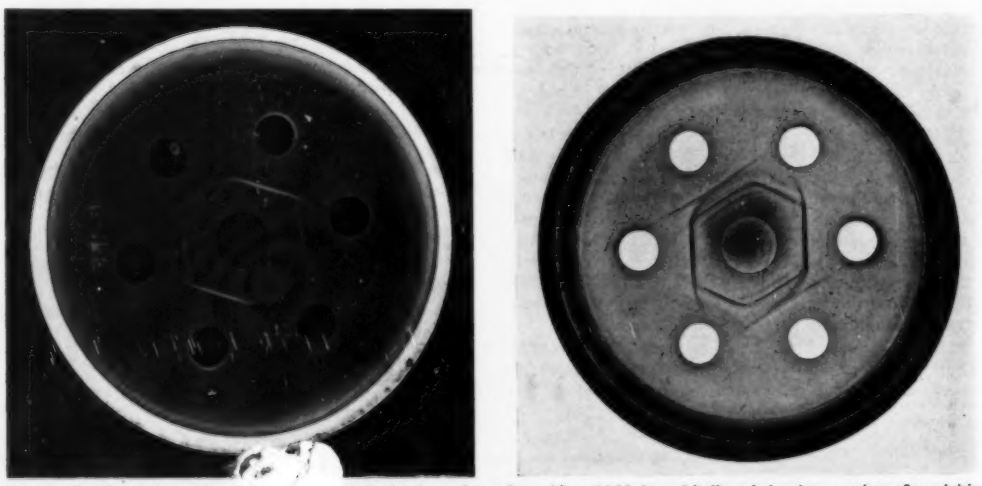
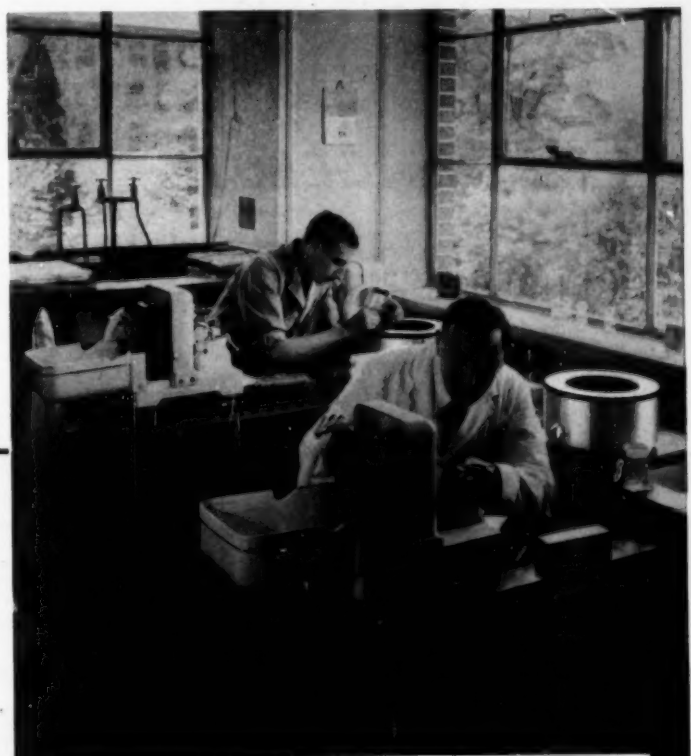


FIG. 1.—Positive and negative pi.c an Ouchterlony plate taken with a shielded pearl bulb and showing a variety of precipitin bands. The central cup cont.. erum and the peripheral cups contain sera from rabbits which had been immunized against various fractions of rat ..m.



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Cytological Preparations from Protein-free Fluids

H. P. PHILIPSEN AND A. C. HUNT
From the Department of Pathology, University of Bristol

(RECEIVED FOR PUBLICATION JUNE 11, 1960)

During an investigation into the forensic aspects of exfoliative cytology the problem arose of making stained preparations of a few cells in a large volume of protein-free fluid. The method described by Powell (1959), in which the cells are centrifuged on to "evo-stik"-coated rectangular coverslips suffers from two disadvantages. First a special centrifuge head has to be made, and secondly the use of "evo-stik" to promote adhesion precludes the application of alcoholic stains and of the usual methods of dehydration and mounting.

The technique to be described does not restrict the use of any staining methods; it is simple and no modification of the centrifuge head is required. The recovery rate of cells is high and the method may therefore have applications in the examination of body fluids in which only small numbers of cells are present.

The apparatus consists of a 3 in. length of glass tubing of 1 in. diameter. An ordinary $\frac{7}{8}$ in. circular coverslip is placed on a rubber bung of appropriate size which is then used to stopper one end of the glass tube. The cells are fixed by adding an equal volume of 4% formaldehyde to the fluid. This is then poured into the tube, which is centrifuged in an ordinary laboratory centrifuge with a swing-out head. The supernatant fluid is removed and replaced by a similar quantity of physiological saline containing 2 drops of bovine albumin per 10 ml. The mixture is shaken and centrifuged again. The supernatant fluid is pipetted off and the residue allowed to dry on to the coverslip overnight, preferably in a desiccator. The addition of the saline and albumin and the subsequent centrifugation may be omitted if a large number of cells are present, as quite good adhesion is still achieved.

The technique may also be used without preliminary wet fixation, with the advantage that albumin may be added directly to the original fluid. One centrifugation is thus avoided and the recovery rate is higher. It has been claimed by Ferreira and Sabbag (1958) that air drying of smears for 24 hours before fixing has no significant deleterious effects on nuclear appearances or cellular morphology and that a correct diagnosis of atypical and malignant cells is still possible. They believe that the results are better after drying for 24 hours than they are after only one hour. Drying may, however, alter the cytoplasmic staining in the Papanicolaou technique and thus interfere with readings of the eosinophilic index.

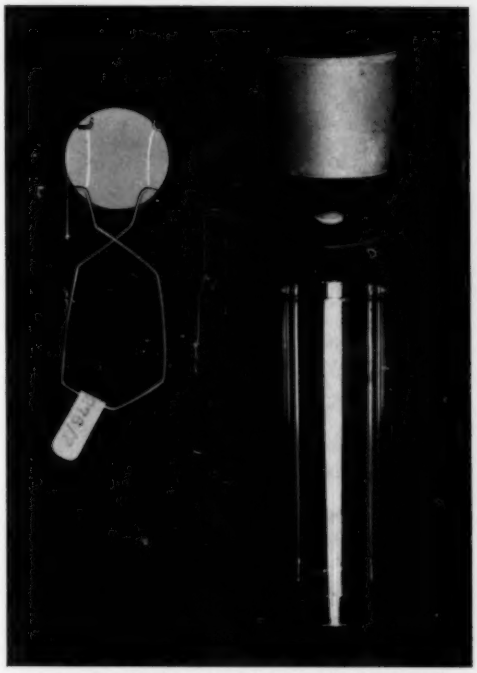

FIG. 1.—Centrifugation tube and clip for holding coverslips.

The staining of circular coverslips in bulk at first presented some difficulty, but this has been overcome by the use of simple holding clips made from stainless steel spring wire of 8 mm. gauge such as is employed in dental practice. The clip is used as a pair of forceps to take the coverslip off the rubber bung, and if always applied in the same way it is obvious throughout the staining process which side of the glass bears the cell smear. Each clip can be labelled individually and any number can be hung side by side in the staining jars on a glass rod passed through the loops.

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Two Useful Modifications for the Seligson Pipetting System

J. T. WEARNE
From the Biochemistry Department, Royal Perth Hospital, Perth, Western Australia

(RECEIVED FOR PUBLICATION MAY 29, 1960)

The advantages of the Seligson pipette in a clinical laboratory are well known (Seligson, 1957; Saifer, Gerstenfeld, and Zymaris, 1958). It is usually attached to a burette for adding diluent. Mather (1960) modifies the stopcock of the pipette and attaches it to an automatic burette. In this laboratory, two devices have proved satisfactory in over two years' continuous service.

(1) A 0.35 ml. Seligson pipette is attached to an "emil" double automatic 25 ml. pipette for preparing plasma dilutions for K^+ estimation. The connexion is made with a short length of polythene tubing, of 2 mm. wall thickness. This makes a joint which is firm, but has some "give" if the pipette is knocked. If the glass tubing is roughened with emery paper there is less danger of it slipping out of the polythene.

A turn of two stopcocks measures the plasma sample and dilutes it without further attention from the technician. The precision is shown in Table I.

TABLE I

(*a*) Mean volume of blood + diluent	= 25·320 ml.
Standard deviation (S.D.)	= 0·063 ,,
Maximum expected error $\left(\frac{3 \text{ S.D.}}{\text{mean}} \times 100\right)$	= 0·75%
(*b*) Mean volume of blood	= 0·341 ml.
S.D.	= 0·0014 ,,
Maximum expected error	= 1·25%
(*c*) Maximum expected error of both operations ($\sqrt{\text{sum of errors squared}}$)	$= \sqrt{0{\cdot}75^2 + 1{\cdot}25^2}$ = 1·46%

(2) In the device illustrated (Fig. 1), the diluent is dispensed by a pipetting syringe.

Both suction and delivery tubes of the Seligson pipette are bent almost at right angles to the calibrated tip. Suction is supplied by a slowly running filter pump. The delivery tube is connected by polythene tubing to a suitable Luer fitting for the pipetting syringe. It is useful to have a polythene cage fitted, as illustrated, to prevent the Seligson pipette being blown out of the connexion by excessive pressure from the syringe. It also gives some protection against knocks.

The apparatus should be loosely assembled at first in order to find the most comfortable operating position. It is not difficult to design a more convenient apparatus, resembling a water pistol, but it would be expensive to construct.

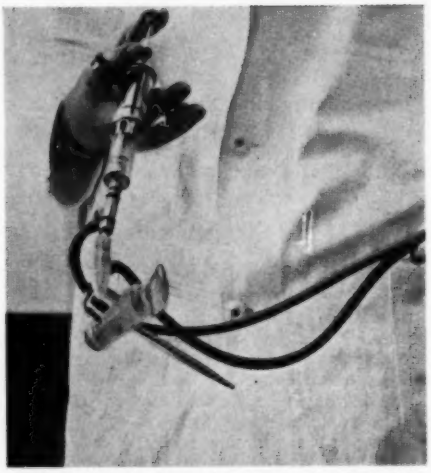

FIG. 1.—A Seligson pipette coupled with a pipetting syringe.

One soon develops a rhythm in using the apparatus. A partly trained technician was timed to prepare 30 samples in nine and a half minutes. In addition, there is no subsequent cleaning of pipettes, as in conventional methods.

TABLE II

(*a*) Mean volume of blood + diluent	= 2·976 ml.
S.D.	= 0·0074 ,,
Maximum expected error	= 0·75%
(*b*) Mean volume of blood	= 0·098 ml.
S.D.	= 0·0011 ,,
Maximum expected error	= 3·3%
(*c*) Maximum expected combined error	= 3·4%

Precision is indicated in Table II. In both cases, 30 samples were taken from a single, well-mixed blood specimen and dispensed, with diluent, into tared volumetric flasks. These were then weighed, to give a measure of the volume of blood plus diluent. The flasks were then filled to the mark with 0.01 M ammonium hydroxide and the haemoglobin content measured in a Hilger "spekker," filter 625. Compared with a dilution of known accuracy, this gave a measure of the volume of blood dispensed. The error introduced by the colorimeter has been ignored.

As standards are run through the same apparatus, it is normally enough to establish consistency, rather than absolute accuracy.

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THE ASSOCIATION OF CLINICAL PATHOLOGISTS

The 65th general meeting was held in London on September 29 and 30, and a joint meeting with the Association of Clinical Biochemists on October 1.

Abstracts of the papers follow:

Investigation of Virus Infections of the Central Nervous System

F. O. MACCALLUM (Virus Reference Laboratory, Colindale) said that a large number of known viruses can attack the central nervous system, sometimes causing inapparent infection, frequently meningitis or "non-paralytic" poliomyelitis, less often encephalitis or encephalomyelitis. Present techniques allow isolation of most of the known viruses more rapidly than tubercle bacilli but less rapidly than meningococci.

The full value of the virus laboratory will only be obtained if possible virus aetiology is considered in each patient investigated in hospital or at home and the appropriate specimens collected in the acute stage of the disease, not after all other tests have proved negative.

As no specific treatment for virus infections is available at present, tests which will identify possible bacterial pathogens obviously take priority, as does examination of the cerebrospinal fluid. If no organism is identified within 24 hours, then specimens for virus isolation and information on amounts of protein and cells in the cerebrospinal fluid, white blood count, temperature pattern, and a few pertinent points of clinical history should be provided for the laboratory. If the onset of the patient's illness is more than three weeks before admission, virus investigations are unlikely to be of value. (For specimens required and methods of investigation see *Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service* (1957) **16**, 151.)

Virus may be found in throat, stools, and cerebrospinal fluid. Stools are the best source of virus in infections with polio (three types), Coxsackie (25–30 types), and ECHO viruses (more than 28 viruses). Throat swabs and particularly cerebrospinal fluid, when available, are necessary for mumps, L.C.M., louping ill, and herpes infections. Serum in the acute stage and 14–21 days from onset should be provided to verify the significance of the virus isolated, or for selected tests when no virus is isolated.

Hospital Conditions as a Factor in Hospital Cross-infection

L. STEINGOLD and L. VOGEL (London) reported that finding causes of hospital cross-infection, and devising methods of correcting faults so found, presented little difficulty. The difficulty lies in introducing such methods into the wards and theatres, and, even more, in seeing that they are continued indefinitely once they have been introduced. Over a whole hospital, this last seems to be impossible.

The reason for this appears to be poor staff morale. Staff are enthusiastic at first, but sooner or later lose interest and revert to their previous unsatisfactory techniques. A possible explanation lies in the conditions under which staff work in most hospitals. Advances in clinical techniques have not been accompanied by the necessary improvements in design, staffing, and equipment of hospitals.

The more obvious parts of a hospital are usually reasonably adequate. Thus, operations are usually performed in a reasonably adequate operating space, and patients are usually accommodated in reasonably adequate wards. The accommodation for other work, however, is often grossly inadequate. Thus, bathrooms, sluice rooms, dressing rooms, changing rooms, and storage facilities may be poor or absent. In addition cleaning and sterilization equipment may be out of date or makeshift.

It is felt that insanitary, unhygienic conditions like these result in insanitary, unhygienic practices.

Control of Staphylococcal Cross-infection in Three Surgical Wards

V. G. ALDER and W. A. GILLESPIE (Bristol) said that cross-infection had been measured in three surgical wards for four years when 1.6% of wounds developed theatre staphylococcal infection, with suppuration. Initially, 40% of open, drained wounds were cross-infected by staphylococci in the wards, as demonstrated by repeated swabbing. Most ward wound infections were clinically "silent." The chief clinical manifestations of ward cross-infection were staphylococcal complications other than wound infection, e.g., pneumonia, enterocolitis, parotitis, furunculosis, from which 0.93% of patients suffered before special precautions were taken. Ward cross-infection was mainly due to multiple-resistant staphylococci.

The precautions tested were:

(*a*) *Against Sources.*—Prophylactic application of neomycin-chlorhexidine cream to noses of all patients, and chemoprophylaxis of open and drained wounds with a neomycin-bacitracin-polymyxin spray.

(*b*) *Against Vectors.*—Disinfection of bedding, bath-water, barbers' brushes, urine bottles, crockery, and nurses' hands.

Simultaneous application of all the precautions, in two wards, reduced open wound cross-infection by about two-thirds, and almost eliminated the other staphylococcal complications (pneumonia, etc.). There was a very marked reduction in multiple-resistant strains.

In the third ward, a comparable improvement was obtained by disinfection of bedding and bath-water, together with nasal and wound chemoprophylaxis. The results suggested that the first three of these measures were of special value, but no single precaution was effective when used alone.

The same precautions were used successfully in outbreaks of staphylococcal infection in four other wards.

Punch Actinomycosis

H. I. WINNER (Charing Cross Hospital, London) described the case of a man who damaged the back of his right index finger hitting someone in the teeth. He was treated with penicillin for four days. Although the skin injury healed, the finger became swollen and painful, and remained so for three months. At the end of this time it became acutely inflamed. Radiographs now showed rarefaction of the head of the proximal phalanx. Open operation revealed much pus in the subcutaneous tissues, containing numerous "sulphur granules" in which *Actinomyces israeli* was identified.

After operation he was treated with penicillin and streptomycin for one week, thereafter with penicillin for four months. Uneventful cure followed.

Four similar cases have been found in the literature.

Investigations of the Serological Specificity of Atypical Acid-fast Bacilli

A. BECK (Paddington General Hospital, London) said that four cultures of photochromogenic mycobacteria which had been isolated repeatedly from patients with a disease resembling tuberculosis were compared serologically with the human tubercle bacillus strain H37Rv and with *M. phlei* and *M. smegmatis*. Antisera against these cultures were examined by the Middlebrook test using P.P.D. fractions of mycobacteria as antigens for red cell sensitization. Titration of these sera revealed marked cross-reactivity. By the use of absorption and inhibition techniques, however, a specific antigen could be demonstrated in photochromogens. In addition they were found to have a common antigen with the human tubercle bacillus strain H37Rv, and a third antigen which is shared by all the mycobacterial species examined.

These results supported the view that atypical acid-fast bacilli represent a special group of mycobacteria which is distinct from both tubercle bacilli and saprophytic mycobacteria.

Acute Mesenteric Lymphadenitis Due to *Pasteurella pseudotuberculosis*

N. S. MAIR and E. M. STIRK (Leicester) said that until a few years ago human infection with *Past. pseudotuberculosis* was generally regarded as rare. Between 1910, when the first case was described by Albrecht, and 1952, only 15 cases, most of them characterized by a severe typhoid-like illness, were reported in the medical literature.

In contrast with this severe but rare type of infection a benign and more frequent form is now recognized. About 170 cases have been reported in Germany and a few cases in Hungary and France. There are no references to similar cases in the English literature.

During the period May to October, 1959, 17 cases of mesenteric adenitis were investigated in Leicester for the presence of both virus and *Past. pseudotuberculosis* infection.

Specimens examined included mesenteric glands, appendix, throat swabs, faeces, and serum. Virus was not isolated from any of the specimens, but evidence of infection with *Past. pseudotuberculosis* was obtained in three patients. *Past. pseudotuberculosis* Type IA was isolated from the mesenteric glands of two of the cases, and all three cases showed serological evidence of infection with the organism. The mesenteric glands of two of the cases showed histological changes characteristic of pseudotuberculosis.

The source of infection could not be found in any of the cases, which did not appear to be epidemiologically related.

The Value of *p*H Adjustment of Urine in Chemotherapy of Urinary Tract Infections

W. BRUMFITT and A. PERCIVAL (London) described a method by which the effect of *p*H on the activity of antibiotics could be rapidly tested. This consists of buffering nutrient agar plates to *p*H 5.5, *p*H 7.0, and *p*H 8.0, and then carrying out sensitivity tests by the impregnated paper disc technique. These tests demonstrated that most antibiotics have an enhanced activity at acid or alkaline *p*H.

Growth, inhibition, and killing curves, carried out in nutrient broth, buffered to the appropriate *p*H levels and containing antibiotics, showed that the results found on solid media could be reproduced in broth.

For most antibiotics the favourable *p*H could be predicted, but not for all.

With the use of a number of harmless drugs, urinary *p*H can be varied over the range *p*H 5.5 to *p*H 8.5. Therefore, a trial was carried out to see whether adjustment of urinary *p*H to the optimum for an antibiotic improved the results of treatment.

Two similar groups of 103 patients, with acute urinary tract infections, were studied. One group was given the antibiotic of choice, based on ordinary sensitivity testing. The other group was given the antibiotic of choice, taking into account *p*H adjustment. In the latter group the urine was adjusted to the optimal *p*H before starting treatment. In both groups a five-day course of therapy was given.

By *p*H adjustment the cure rate (based upon bacteriological examination) was increased from 67 to 88%.

Finally, by *p*H adjustment and chemotherapy in 156 patients with acute urinary tract infections who had relapsed, cure was obtained in 125 (80%).

It is concluded that *p*H adjustment of the urine, combined with chemotherapy, gives improved results in the treatment of acute urinary tract infections, and

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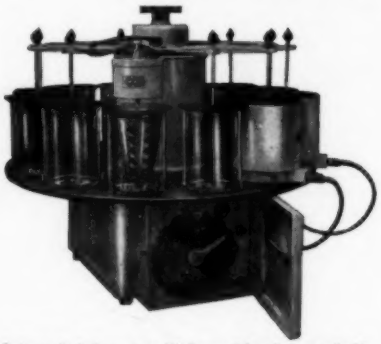

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perhaps reduces the incidence of subsequent chronic pyelonephritis.

The Role of the Catheter in Causing Urinary Tract Infection in Pregnancy and the Puerperium

B. I. DAVIES and W. BRUMFITT (London) reported that in order to study the role of the catheter in causing infection 276 maternity patients were divided into three groups according to their catheterization history.

Group I contained 105 patients who had normal deliveries and were not catheterized.

Group II contained 66 patients who also had normal deliveries, but on whom a catheter was passed once.

Group III was composed of 105 patients in whom complications in labour necessitated catheterization.

Only patients whose urines on admission to hospital were uninfected were included in the series, and any subsequent infections were detected by frequent bacteriological and cytological examination of mid-stream urine specimens while they were in hospital, and, in addition, one month after discharge.

The sole criterion of infection was a urine bacterial count in excess of 100,000 organisms per millilitre. Such counts were usually, but not always, accompanied by leucocyte counts of 50 per c.mm. or more. Many infections were asymptomatic and would not have been suspected clinically.

The infection rates were: Group I, five out of 105 (4.75%); Group II, six out of 66 (9%); Group III, 24 out of 105 (22.8%). These results show that catheterization of the normal patient approximately doubles the risk of infection, whereas the complications of labour which compel the clinician to catheterize (Group III) greatly increase this danger. Certain of the reasons for catheterization carry an especially high risk, such as catheterization for retention of urine (40.6% infected), or investigation of puerperal pyrexia (36.4% infected), whereas catheterization preceding forceps delivery does not greatly enhance the danger (12.5% infected).

Of the 37 infections, 31 were caused by *Bact. coli*, the organism most commonly associated with primary, non-obstructive urinary tract infection.

Further Observations on Tumours of Salivary Tissue

J. MALCOLM CAMERON (Southern General Hospital, Glasgow) said that in a series of 401 examples of tumours of salivary tissue there were 315 cases of pleomorphic adenomata constituting 78.5% of the total. In addition there were 64 cases of carcinoma (16%) and 16 cases of adenolymphoma (4%).

The parotid gland accounted for just over 80% of the pleomorphic adenomata, 53.6% of which occurred on the right side, and there appeared to be a slight preponderance of females. Almost 60% occurred before the age of 40 years and 90% before 60 years of age. Most tumours grew slowly and intermittently and recurrences were noted in 30% following surgery.

Carcinoma occurred mainly between the fifth and eighth decade and again the parotid gland was the commonest site. The recurrence rate was much higher. All but one of the adenolymphomas occurred in the parotid gland, the one exception being in the right tonsil, bilateral adenolymphomata being found in four patients.

Attempts to produce tumours in experimental animals, similar to human pleomorphic adenomata, were described. In general these attempts were unsuccessful, as have been those of previous workers.

A Study of the Post-natal Anaemia in Erythroblastosis

P. S. MACFARLANE, A. L. SPEIRS, and J. W. WHITELAW (Stirling) said that a series of 30 cases of neonatal anaemia due to Rh incompatibility, and including all grades of severity, had been studied. Full blood examinations were frequently performed and in most cases three specimens of bone marrow were examined.

It was concluded that although excessive red cell destruction is the prime cause of the anaemia another important aetiological factor is the inability of the marrow to produce red cells in the neonatal period. This is essentially a physiological condition, but it is acknowledged to be a significant factor in the causation of the anaemia of prematurity.

It seemed possible that the marrow inactivity might be due to nutritional deficiency and folic acid treatment was tried with only limited success. The alternative explanation is that at birth, when the marrow is invariably responding fully to the haemolysis, there is present in the baby's blood a marrow-stimulating factor which later becomes deficient.

To test this hypothesis the Rh antibodies were absorbed from plasma obtained at exchange transfusion and the plasma was administered intravenously during the phase when red cell production was in abeyance. Significant reticulocyte responses were obtained. Similar results have been obtained using fresh frozen plasma.

These studies offer evidence that an important factor in the cause of the anaemia in erythroblastosis is deficiency of an erythropoietic stimulating factor.

The Practical Value of an Electronic Blood Cell Counter in a Routine Laboratory

C. H. WRIGLEY (Winchester) reported on an electronic blood cell counter installed two years ago at the Winchester laboratory. The machine is of the fluid flow type, first developed by Crosland-Taylor and later manufactured under the trade name E.E.L.

It was not considered that an electronic counter for counting red cells only was financially justifiable in a routine laboratory.

There had been two main difficulties in using the machine for counting leucocytes, but these had now been overcome.

The difficulties were: (*a*) The mechanical design of the jet chamber originally made adjustment of the jets difficult, but this had now been remedied by the manufacturers; (*b*) precipitation of a protein-like substance, which caused frequent blocking of the jets. This was overcome by adding ammonia to the circulating fluid, making the concentration 0.04%.

During the last 12 months this machine regularly averaged 25 leucocyte counts per day. The time saved by the machine more than compensated for that spent on routine maintenance. Its great value is in relieving the technicians of the fatigue of visual counting.

It is considered that any laboratory carrying out more than 15 white cell counts on an average per day will benefit from one of these machines.

The Adhesiveness of Platelets

J. R. O'BRIEN (Portsmouth) described the following methods in the investigation of the adhesiveness of platelets in native whole blood to various surfaces:

Adhesion to Damaged Cells.—(*a*) The Ivy bleeding time (which may be influenced by other factors as well as platelet adhesiveness); (*b*) tissue cultures of human amnion cells grown in sheets were damaged by heating. Native blood was trickled over these cells and the number of platelets adhering was counted.

Adhesion to Glass.—Native whole blood was forced at a constant rapid speed through a tube packed with glass beads. The decrease in the platelet count of blood issuing from the beads compared with an initial count was a measure of how many platelets had stuck.

Aggregation.—Aggregation of one platelet to another to form clumps in native plasma was observed visually.

The platelets in blood from heparinized and "dindevan"-treated patients and from all the coagulation defects studied adhered normally to glass, and these patients have a normal bleeding time.

The following substances inhibited all three test systems *in vitro*: Cocaine, benadryl, quinine, cetyl tetra-ammonium bromide, cetyl pyridinium bromide (both positively charged detergents), and sodium citrate. The first two systems only were inhibited by sodium salicylate. Cocaine, benadryl, and quinine did not interfere with the clotting mechanism.

It was concluded that platelet aggregation is probably essential for clot retraction; that platelet adhesion is calcium dependent; that clotting and adhesion to cells or to glass are independent phenomena; that at least part of the mechanism of aggregation, which takes time, is different from that of immediate adhesion; that the adhesion of platelets in whole blood to glass and to damaged cells can be prevented by some chemicals. Those chemicals investigated were actively anti-adhesive *in vitro* only at concentrations that would be lethal *in vivo*. Since the rapid adhesion of platelets to "damaged" endothelial cells is probably an essential step in thrombus formation, it is possible that a harmless drug with anti-adhesive properties might prevent thrombosis.

Indigenous Amoebiasis

W. P. STAMM (R.A.F., Halton) drew attention to the chronic insidious nature of the clinical symptoms of the majority of cases of intestinal amoebiasis and to the further difficulty in diagnosis caused by an incidence of carriers of *E. histolytica* in the United Kingdom between 5 and 10%.

He thought that the fact that civilian routine laboratories did not report such a high incidence was due to direct microscopy of faeces not being practised as a routine and to inexperience in faecal protozoology of pathologists and technicians.

He emphasized the difficulties in identification of *E. histolytica* and illustrated with lantern slides appearances of *E. histolytica* when stained in faecal smears and histological sections by some of the methods most suitable for routine use.

He drew attention to amoeboma in the differential diagnosis of abdominal tumours, and warned against operating on a patient who had been overseas and presented with an abdominal tumour, without a careful examination of stool specimens.

He concluded by pointing out that although clinical amoebiasis was undoubtedly commoner in the United Kingdom than was generally recognized, symptomless infections were even more common and a zealous search for amoebae not followed by good clinical judgment could easily result in amoebophilia amongst doctors and a consequent amoebophobia in their patients.

Eosinophilia and Tropical Disease

J. K. MASON (R.A.F., Halton) said that many of the conditions characterized by tropical acquisition, a prolonged course, and an associated eosinophilia, presented with vague clinical symptoms and might often be first indicated in the laboratory.

The finding of an eosinophilia in a person returned from overseas does not necessarily imply a tropical origin but should always excite a search for such a cause. The most frequently responsible organisms are the helminths. Of the intestinal nematodes, ascaris, ankylostoma and strongyloides were discussed in relation to symptoms and the occurrence of eosinophilia.

Infestation with filarial worms gives rise to a profound eosinophilia. Common methods of presentation in Europeans were described.

The occurrence of schistosomiasis in Europeans was discussed. The diagnosis should be made in all persons returning from an endemic area presenting with haematuria until it can be disproved. The diagnosis of the acute and chronic disease was discussed.

Tropical eosinophilia was described as a differential diagnosis of other chronic pulmonary diseases.

The frequent difficulty of demonstrating the causal organism in a case of unexplained eosinophilia was stressed and a plea made for a very thorough parasitic investigation in every case.

Pyrexia of Unknown Origin of Tropical Origin

R. MORTIMER (R.A.F., Halton) said that the most important tropical disease likely to be encountered in the U.K. is malignant tertian malaria, which in its pernicious forms, particularly cerebral malaria, is still killing patients. The possibility of malaria should always be considered in any patient with a fever who had arrived in the U.K. from the tropics via central Africa about 10 to 14 days previously, with perhaps a history of an unscheduled night stop en route. There is usually no difficulty in demonstrating parasites in blood films in such cases, and, if found, the results should be phoned at once to the ward so that treatment can begin with the minimum delay. All patients with pernicious malignant tertian malaria should be treated as emergencies with intravenous or intramuscular chloroquine or intravenous quinine. Delay in treatment or treatment by mouth may result in tragedy. Case histories were described of deaths due to the various failures of diagnosis, treatment, and management, and the recognition of other types of malaria was discussed. Emphasis was placed on the need to think in terms of multiple diagnoses in relation to tropical diseases, and the presentations of kala azar, relapsing fever, brucellosis, and amoebic liver abscess and their clinicopathological diagnosis, were discussed. The pitfall of not considering syphilis, which in the tropics is often florid in character, was mentioned. In conclusion a plea was made for a more positive approach to the possibility of tropical disease occurring in patients in the U.K.

Diseases Encountered in Immigrants

K. R. HILL (Royal Free Hospital, London) said that, of 250,000 immigrants in the U.K., more than half were West Indians. Two main aspects of their background are (1) racial pattern, and (2) dietary malnutrition.

Negroes are preponderant but are mixed with Anglo-Saxon, Celtic, Chinese, East Indian, and Mediterranean immigrants. Sickle cell disease is present in 5 to 10% of the populace. Sickle cell anaemia (genotype S.S.) is easily recognized, but the trait (S.A. or S.C.) may be complicated by other haemoglobinopathies. All immigrants should have their blood tested for sickling.

Leukaemias, aplastic, hypochromic, and megaloblastic anaemias are common, but pernicious anaemia is rare. (This last statement may not be valid.)

Dietary malnutrition is reflected in kwashiorkor and hepatic veno-occlusive disease. Cirrhosis is shown in 3 to 4% of necropsies, and primary hepatic carcinoma associated with cirrhosis in 16% of all primary carcinoma.

Tuberculosis is very common and fungus diseases may be encountered.

Syphilis and gonorrhoea are very common; S.T.S. is positive in 30% of hospital cases. The early stages of yaws are unlikely to be encountered, but the later stages of scarring and deformity may cause confusion.

The Negro has a fibrous diathesis and there is a high incidence of keloids, fibromyomata, and osteogenic fibromata of the jaw.

Lymphogranuloma venereum is common and the L.G.V.C.F.T. is positive in 30%. This test, and S.T.S., should be carried out in all immigrants.

Atherosclerosis, particularly of the coronary arteries, is common; myocardial infarction is uncommon. Hypertension is very prevalent and this is associated with a high incidence of nephritis. Streptococcal infections are prevalent and rheumatic fever not uncommon.

The West Indian does not withstand operative trauma very well and post-operative circulatory collapse is not uncommon. This may be related to the fact that the adrenals (cortices) are about two-thirds those of Europeans. At operation there is often a hyperglycaemia which may be related to an unstable carbohydrate metabolism. A special Jamaican type of diabetes has been described. Malaria may be encountered, as may be microfilaria, as an incidental finding in the blood.

Pathogenesis of the Malabsorption Syndrome

A. C. FRAZER (The Medical School, Birmingham) said that the primary aetiological factor in the malabsorption syndrome may be faulty intraluminar preparation for absorption, due to lack of pancreatic enzymes or bile salts, or incoordination of gastrointestinal function. A secondary enteropathy may be superimposed in such patients, due to electrolyte imbalance or bacterial infestation. A primary enteropathy may arise from gluten intolerance, folic acid deficiency, bacterial infestation, or small intestinal disease, or to a combination of two or more of these factors.

A deleterious agent can be demonstrated in wheat gluten which inhibits small intestinal motility by interference with acetylcholine release. This substance is ultrafiltrable, heat-stable, resistant to peptic/tryptic digestion, but its action is destroyed by incubation with small intestinal mucous membrane extract. Gluten or appropriate fractions cause deterioration in patients in remission on a gluten-free regimen. Gluten intolerance may be a major or a secondary aetiological factor.

Folic acid deficiency is an important feature of sprue; the cause of the folic acid deficiency has not yet been satisfactorily established. Folic acid deficiency may occur as a secondary complication of gluten-induced enteropathy.

Bacterial infestation of the small intestinal lumen may be a major factor, as in gastro-colic fistula and blind-loop syndromes; it may also play an important part in other forms of the malabsorption syndrome.

Small intestinal disease, such as regional enteritis, lymphatic obstruction, or Whipple's disease, may be associated with the malabsorption syndrome.

Whatever the cause of the malabsorption, unabsorbed food materials pass into the lower reaches of the bowel and give rise to increased bacterial

activity. The flora modify many of the faecal constituents. In some cases significant amounts of fat and nitrogen that are not of direct dietary origin may be found in the stools.

The Use of Fat Labelled with ^{131}I in the Diagnosis of Steatorrhoea

M. LUBRAN (West Middlesex Hospital, London) said that absorption of fat from the intestine could be investigated satisfactorily by measuring the faecal radioactivity for four days after the oral administration of 10 μc. of ^{131}I-triolein. Of 183 tests carried out simultaneously with a six-day chemical fat determination, complete agreement between the two tests was found in 139 (76%) of tests and borderline agreement in a further 44 (13.7%). The radioactive and six-day chemical fat tests are about equally effective in detecting steatorrhoea. However, because of the fluctuations in the faecal excretion of fat in steatorrhoea, it may be necessary to carry out daily faecal fat measurements for a prolonged period. Radioactive fats are not suitable for long-continued measurements.

Blood levels of radioactivity following oral ^{131}I-triolein are not a satisfactory guide to steatorrhoea because of the wide range of values in normals and the normal values obtained in some patients with steatorrhoea. Urinary radioactivity measurements are also unsatisfatcory in the diagnosis of steatorrhoea. Administration of ^{131}I-oleic acid and measurement of blood radioactivity after a previous abnormal curve with ^{131}I-triolein may be of limited value in the diagnosis of pancreatic disease. Simultaneous administration of ^{82}Br-oleic acid and ^{131}I-triolein and the study of the ratio of ^{82}Br to ^{131}I in the blood two, four, and six hours later provides a promising test of pancreatic function.

The d-Xylose Excretion Test and its Use in the Diagnosis of Malabsorption

W. T. COOKE and DOROTHY FOWLER (Birmingham) said that the five-hour urinary excretion of d-xylose after the oral ingestion of 25 g. of d-xylose was a useful test in the diagnosis of disorders of the small intestine. Their mean value for 37 fasting normal subjects under the age of 65 years was 7.2 g. (S.E. 0.27) and for nine normal subjects over 64 years 2.9 g. (S.E. 0.10). Thus, a low result in patients 65 years and over is of little diagnostic significance. The normal results of the other workers were shown and the possible reasons for the discrepancies found discussed. The lower limit of normal was set at 4.2 g. d-xylose in the five-hour urine, i.e., 7.2–2.0 S.D. Results from 194 patients were presented. Of these, 49, under 65 years of age, had xylose excretions of less than 4.2 g. Thirty-two of the 49 were "idiopathic steatorrhoea," most having been confirmed by jejunal biopsy. The 17 remaining patients under 65 years of age who had five-hour urine xylose excretions less than 4.2 g. were discussed. Two were aged 61 and 64 years respectively, another was suffering from tropical sprue, one had a past history of steatorrhoea, and four were excreting between 3.8 and 4.0 g. xylose. Fourteen patients with "idiopathic steatorrhoea" had five-hour d-xylose excretion results of 4.2 g. and over. Of these, five had been on gluten-free diets for one year or more and had responded well. The other nine were discussed. Of 15 patients with pancreatitis, only one, a woman of 68 years, excreted less than 4.2 g. Results from patients with various other gastro-intestinal disorders were discussed.

Comparisons of five-hour urine xylose excretions with faecal fat excretion and folic acid excretion tests were presented.

Total Body Electrolytes in Intestinal Disease

J. P. NICHOLSON and J. F. ZILVA (Westminster Hospital, London) had measured the total exchangeable sodium and potassium values in 19 patients suffering from intestinal diseases, particularly ulcerative colitis. The experimental method and determination of normal values were described.

Generally speaking, the exchangeable sodium values were within the normal range whilst the exchangeable potassium values were well below the normal limits.

The picture is complicated by the fact that steroids are frequently used in the treatment of these conditions.

Further information may be obtained by considering the two values in conjunction, i.e., the sum of sodium and potassium, and their ratio.

The mechanisms involved were discussed and some suggestions put forward as to the way in which the potassium loss might be minimized.

Blood Volume and Plasma Protein Levels Before and After Gastrectomy

ALEXANDER SWAN, GEOFFREY T. ALLEN, and NORMAN C. TANNER (London) said that blood volumes were estimated by the dye method using Geigy blue pre-operatively, nine or ten days post-operatively, and again six or seven months after operation, in a series of cases of partial gastrectomy and vagotomy and a small number of those of pyloroplasty and radical gastrectomy. Plasma proteins were also determined in a large proportion of these cases. The following trends were observed.

(1) Patients with peptic ulcer are, as a rule, underweight and show a deficit of blood volume which is due more to shortage of red cells than to that of plasma.

(2) The red cell deficit is further aggravated by the operative blood loss and tends to persist, being still demonstrable six to seven months after operation. Patients who received blood transfusion (two pints on the average) at or immediately after partial gastrectomy showed no appreciable reduction of the total red cell volume (T.R.C.V.) after operation as well as higher T.R.C.V. levels than cases operated on without blood transfusion six months after.

(3) Haemoglobin and haematocrit values do not adequately reflect either the pre-operative T.R.C.V. deficit or its post-operative variations, at times showing

a change in the opposite direction to that of T.R.C.V.

(4) A small number of cases with the dumping syndrome gave no reason for belief that this condition is associated with a reduced blood volume.

(5) Plasma globulins rose after partial gastrectomy (10 days after). This was accompanied by a marked fall in the albumin fraction, so that the total plasma proteins fell. Six months after operation the total albumin circulating mass tended to rise to levels exceeding the pre-operative, whereas the globulins dropped almost to the pre-operative state. This was not reflected in the protein concentration figures, those of the albumin remaining far below the pre-operative levels.

Separation and Determination of Some Basic Substances in Urine by Ion Exchange Chromatography

S. TOMPSETT (Edinburgh) said that the cation exchange resin, "dowex 50 × 12," has been used for the separation of a number of basic substances from urine as a preparatory to colorimetric analysis or identification by paper chromatography. Concentration may be achieved, and by using gradient elution (varying concentrations of hydrochloric acid) specificity is increased. Some reference was also made to elution by ammonia.

The use of the anion exchange resin, "dowex 1," for the separation of some basic substances from urine was also referred to.

A New Approach to the Estimation of Ammonia in Blood

J. C. B. FENTON (St. Bartholomew's Hospital, London) gave a brief review of the analytical techniques used for blood ammonia estimation. Almost all methods used for the isolation of ammonia depend upon the volatility of this compound in alkaline solution. It is generally agreed that some ammonia is falsely produced under these conditions, and it has never been positively shown that the ammonia estimated in blood is not entirely an artefact derived from the alkaline hydrolysis of unstable nitrogenous substances. The lack of agreement as to what constitutes a normal value reflects the generally unsatisfactory state of the topic.

An alternative technique which circumvents some of these objections was put forward. This employs ion exchange at a neutral *p*H of separated heparinized plasma. The ammonium ion can be isolated by adsorption on to a sulphonic acid type ion exchange resin in the sodium cycle and eluted by a strong solution of sodium chloride. After treating the eluate with aluminium hydroxide the ammonia content is estimated by means of an indophenol blue colour reaction. The technique is sensitive and levels down to 10 μg. NH_3 nitrogen/100 ml. can be estimated with ease.

The whole process of separation of the plasma and ion exchange is carried out under ice-cold conditions. Under these circumstances no rapid rise in the ammonia content of plasma over the course of several hours has been found. Normally the ammonia content of plasma appears to be less than 50 μg./100 ml.

Lipaemia, Coagulation, and Thrombolysis in Ischaemic Heart Disease

D. C. O. JAMES, J. DRYSDALE, J. D. BILLIMORIA, and N. F. MACLAGAN (Westminster Hospital, London) reported comparisons of lipaemia, coagulation, and thrombolysis made between 28 normal subjects, 24 with angina pectoris and 34 with myocardial infarction. All subjects were in the age group 40 to 70 years. The normals were healthy subjects showing no evidence of ischaemic heart disease, hypertension, nephritis, diabetes mellitus, xanthomatosis, or thyroid disease and all had a normal E.C.G. The tests were carried out under standard conditions, with and without inclusion of butter in the test breakfast.

Under fat-free conditions the lipaemia in normal males under 40 years of age was significantly higher than in normal females under 40. Significant differences from normal were noted in the pathological groups; thus the angina pectoris group showed a higher lipaemia, a shorter Stypven time, and a longer thrombolysis time, while the myocardial infarction group showed similar trends but the differences from normal were less significant.

The differences due to inclusion of 1½ oz. of butter in the test breakfast were not sufficiently significant to justify the use of fat-feeding tests in the investigation of ischaemic heart disease.

The effects of anticoagulants (phenylindanedione) were studied, and these caused a decrease in the lysis time. The time interval between myocardial infarction and the tests did not seem to influence the results.

The results as a whole indicate some disturbance of plasma lipids and of coagulation and thrombolytic mechanisms in ischaemic heart disease which were much more significant in angina pectoris than in myocardial infarction.

17-Hydroxycorticosteroid Excretion in Patients with Peptic Ulceration

A. G. GREEN and C. N. PULVERTAFT (York) described how, using a modified Appleby, Gibson, Norymberski, and Stubbs technique, the excretion of total 17-hydroxycorticosteroids (17-HCS) had been studied in patients suffering from peptic ulceration and in normal controls.

The mean excretion of 17-HCS for 90 men with active duodenal ulcers was found to be 8.8 mg./day, significantly lower than that of 52 normal men (11.6 mg./day), the age distribution for these groups being very similar. Twenty-eight men with active gastric ulcers had a mean output of 7.8 mg./day; 40 men who had undergone partial gastrectomy for duodenal ulcer some 10 to 15 years previously showed a mean output of 7.8 mg./day; the mean for 29 men with duodenal ulcers in a quiescent phase was again 7.8 mg./day. For these three groups the mean age was slightly higher than that of the controls and the active

duodenal ulcer group. Very little difference was found in the excretion of 17-ketosteroids between the control and duodenal ulcer groups. The excretion of 17-ketosteroids in the gastric ulcer and post-gastrectomy groups was slightly lower than in the control group.

In the active ulcer group a definite correlation was noted between 17-HCS excretion and the volume of urine (D.U. $r=0.52$; G.U. $r=0.49$). A similar correlation was found for the post-gastrectomy group ($r=0.54$). This finding was not observed in the controls ($r=0.11$) and the quiescent ulcer group ($r=0.23$).

The excretion of 17-HCS was estimated before, and six months after, partial gastrectomy for peptic ulceration, in 18 men. In 13 of these the post-operative excretion was less than the pre-operative level, the mean falling from 9.9 to 6.9 mg./day. A similar observation for 17-ketosteroid excretion was noted in 23 out of 28 cases, the mean falling from 11.4 to 8.3 mg./day. This fall was not observed after vagotomy and gastroenterostomy (23 cases) when there was very little difference in the pre- and post-operative levels.

Experience with the Estimation of Serum Protein-bound Iodine

D. R. C. WILLCOX and W. G. KING (Bethlem Royal and the Maudsley Hospital, London) gave an account of the use and control of a method of estimation of serum protein-bound iodine (P.B.I.) slightly modified from that of Grossman and Grossman (1955). The results over a period of five years were summarized and their clinical significance discussed.

Precautions against contamination and the advantages of universal containers for the ashing stage were described. The effect of various ashing temperatures and of subsequent complete solution of the ash was shown. The use of standards carried throughout the whole procedure was considered essential. Recovery experiments estimated 95–98% of thyroxine added to serum precipitates. In routine use consistency of results was further checked by repeating one estimation from the previous batch on each occasion. The S.D. of the differences between such repeated estimations is 0.44 μg. per 100 ml.

In considering the results of some 2,000 duplicate estimations, 51 normal subjects gave a range and mean in close agreement with those originally described. It was shown that the distribution of values in euthyroid psychiatric and in euthyroid general hospital patients differs little from the normal, although there is a very small overlap with the hypothyroid and hyperthyroid groups, which are clearly separated from the normals. The general stability of thyroid function was demonstrated in serial studies, but some exceptions were quoted. The relationship to adrenocortical function and to mood or psychiatric diagnosis was considered. The results from 60 carcinomatosis patients submitted to hypophysectomy were also discussed.

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NOMENCLATURE OF ABNORMAL HAEMOGLOBINS

In the course of the VIIIth International Congress of Haematology in September, 1960, in Tokyo, Japan, 44 members met to discuss the nomenclature of the human haemoglobins. This field is expanding so rapidly that it is not possible even at an international congress to obtain a full representation of all disciplines and groups of workers. The absence of several distinguished investigators in this field was acutely felt. Nevertheless the VIIIth International Congress of Haematology was considered the best possible occasion for such a gathering. The following recommendations are being sent to appropriate journals so that any of the distinguished investigators not present will be able to criticize them in public.

(1) The recommendations on nomenclature made at the Symposium on Abnormal Haemoglobins in Istanbul, 1958, and published in 1959 (Council for International Organizations of Medical Sciences, 1959), are endorsed and the letters A–N (with the exception of B), and S are recognized as naming haemoglobins as there defined.

(2) The description of the variants of haemoglobin M as M_B, M_M, M_S (from Boston, Milwaukee, and Saskatoon respectively) is accepted (Gerald, 1958; Pisciotta, Ebbe, and Hinz, 1959), and it is suggested that new haemoglobins M are described with fully subscripted names until they have been shown to differ from these three, when they should be given subscript initials (M_{Iwate}, possibly later M_I).

(3) The letters O, P, and Q are being allotted to the haemoglobins described under these letters (Lie-Injo Luan Eng and Sadono, 1958; Schneider and Haggard, 1958; Vella, Wells, Ager, and Lehmann, 1958).

(4) Until the next meeting of the International Congress the letters R–Z (excepting S) should not be allotted to new abnormal haemoglobins but these should be given names of localities. It should be left to the individual workers to choose the most meaningful name from the origin of the propositus, or the laboratory, hospital, town, or district where the haemoglobin was found. A new name should not be allotted in this way unless it has been ascertained that the haemoglobin to be named is different from all those adequately described in the literature.

(5) Of the two designations of the haemoglobin A_2 variant: A_2' and B_2 (Kunkel, Ceppelini, Dunn, and Firsheim, 1959), the first is found more acceptable. If a third variant should be found it should be named A_2'' and not C_2.

(6) The names of the three known peptide chains of human haemoglobin are α, β, and γ (Schroeder, 1959), and it is suggested that the normal chains should be designated in that way, i.e., not α^A or α^F, β^A and γ^F. The genetic superscript for the normal gene product $^+$ (α^+, β^+, γ^+) will not be used as it implies to the chemist a positive charge.

(7) The expressions β_4 for H and γ_4 for Bart's may be used when their identity is fully accepted. However, until the next meeting of the International Congress the traditional names should be mentioned at least once in each publication.

(8) The present custom to describe an abnormal chain by adding the name of the appropriate abnormal haemoglobin in superscript should be maintained ($S = \alpha_2\beta_2^S$; Hopkins-2 $= \alpha_1^{\text{Hopkins-2}}\beta_2$).

(9) A polypeptide chain should not be designated with a new small Greek letter (such as δ, ϵ, etc.) until chemical evidence for complete separate identity from the α, β, and γ chains (such as exists between these chains themselves) has been established, genetic differences notwithstanding.

(10) It is expected that the analysis of the amino-acid sequences in the globin molecule will eventually be followed by a precise chemical nomenclature. Meanwhile if a haemoglobin has been identified by the usual methods of electrophoresis, chromatography, spectroscopy, alkali denaturation, cold denaturation, and solubility tests, it should be described by the accepted capital letter as hitherto (S, C, D, G, E, etc.) as recommended by the working party meeting at the VIth International Congress, Boston, 1956 (Lehmann, 1957). If comparison has been made on the same lines and with the same completeness with a haemoglobin carrying the name of a locality, that name should be applied. If the abnormal chain is identified this should be indicated by a subscript, for example, D_α, D_β, and until the full identity by examining the amino-acid sequences has been established this subscript should be followed by a declaration of origin ($D_{\beta\ \text{Los Angeles}}$, $G_{\alpha\ \text{Ibadan}}$). This implies that $D\gamma$ will have to be renamed $D_{\beta\ \text{Punjab}}$. If two or more of such haemoglobins are then found identical by analysis of the amino-acid sequences, only the name of the haemoglobin which was first discovered and fully defined by the conventional methods should be retained. For example, if $G_{\alpha\ \text{Ibadan}}$ and $G_{\alpha\ \text{Azumakoli}}$ are found to be identical regardless of which the amino-acid sequence has been fully examined first $G_{\alpha\ \text{Ibadan}}$ should be the remaining name. However, until the next meeting of the International Congress the alternative names should be mentioned at least once in each publication.

(11) No generally acceptable name was agreed upon for the familial condition in which haemoglobin F persists into adult life without morphological changes

of the red cells and without anaemia. The term non-microcythaemic thalassaemia was considered not specific enough as it might equally apply to the familial condition where haemoglobin A_2 is raised without associated morphological changes. If the expression "high F gene" is used this should be done with reservation and only provisionally until further knowledge allows better terminology.

(12) If several haemoglobins are present the phenotype should be designated by listing the haemoglobins in order of decreasing concentration regardless of genetic considerations (sickle-cell trait=AS, sickle-cell anaemia=SF, sickle-cell thalassaemia=SAF, or SFA, etc.,).

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13	The Identification of Serotypes of *Escherichia coli* Associated with Infantile Gastro-enteritis	1956	Joan Taylor
14	The Determination of Serum Iron and Serum Unsaturated Iron-binding Capacity	1956	Arthur Jordan
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22	Investigation of Haemolytic Anaemia	1959	J. G. Selwyn
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25	Titration of Antistreptolysin O	1959	H. Gooder and R. E. O. Williams
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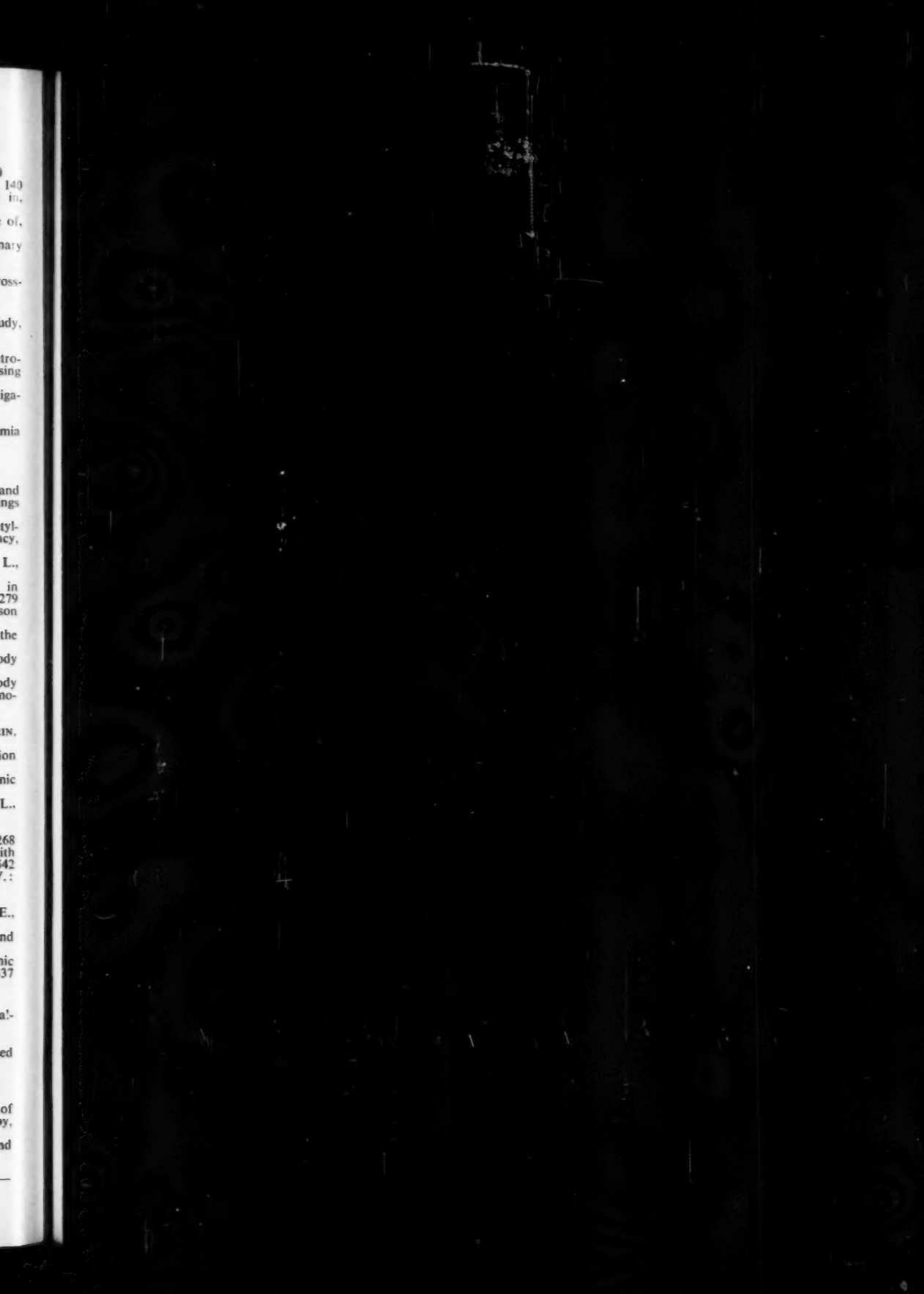
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